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VOLUME ~~XV~~ —



1947-18

ACADEMIC PRESS INC. PUBLISHERS  
NEW YORK, N. Y.

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## CONTENTS OF VOLUME 15

No. 1, OCTOBER, 1947

K. P. DIMICK, G. ALDERTON, J. C. Lewis, H. D. LIGHTBODY AND H. L. FEVOLD. Purification and Some Properties of Subtilin	1
R. E. FEENEY, H. D. LIGHTBODY AND J. A. GARIBALDI. Zinc as an Essential Element for Growth and Subtilin Formation by <i>Bacillus subtilis</i> .....	13
GRACE MEDES, DOROTHY C. KELLER AND AMY KURKJIAN. Essential Fatty Acid Metabolism. I. Essential Fatty Acid Content of Rats on Fat-Free and Pyridoxine-Free Diets....	19
A. R. WHITEHILL, J. J. OLESON AND Y. SUBBAROW. A <i>Lactobacillus</i> of Cecal Origin Requiring Oleic Acid.....	31
OTTO H. MÜLLER AND JOHN STAIGE DAVIS, JR. Polarographic Studies of Proteins and Their Degradation Products. II. Normal Values of the "Protein Index".....	39
WALTER KOCHOLATY AND RENATE JUNOWICZ-KOCHOLATY. The Use of Cation Exchangers for the Concentration and Purification of Antibodies of Basic Nature.....	55
FRED H. MATTSO, JOHN W. MEHL AND HARRY J. DEUEL, JR. Studies on Carotenoid Metabolism. VII. The Site of Conversion of Carotene to Vitamin A in the Rat.....	65
CATHERINE ELISABETH WIESE, JOHN W. MEHL AND HARRY J. DEUEL, JR. Studies on Carotenoid Metabolism. VIII. The <i>in vitro</i> Conversion of Carotene to Vitamin A in the Intestine of the Rat.....	75
SEDGWICK E. SMITH AND G. H. ELLIS. Copper Deficiency in Rabbits. Achromotrichia, Alopecia, and Dermatitis.....	81
THOMAS D. FONTAINE, ROBERTA MA, JANET B. POOLE, WILLIAM L. PORTER AND JOSEPH NAGHSKI. Isolation of Rutin from Tomatin Concentrates.....	89
FREDERICK KAVANAGH. Chemical Determination of Pleurotin, an Antibacterial Substance from <i>Pleurotus griseus</i> .....	95
W. H. McSHAN, ROLAND K. MEYER AND W. F. ERWAY. Effect of Estrogens and Androgens on the Succinoxidase System of Rat Tissues.....	99
F. ROSEN AND WM. A. PERLZWEIG. On the "Pellagrigenic" Inactivity of Indole-3-Acetic Acid in the Rat.....	111
MAX A. LAUFFER AND W. C. PRICE. Electrophoretic Purification of Southern Bean Mosaic Virus.....	115
NOEL H. GROSS AND C. H. WERKMAN. Isotopic Composition of Acetylmethylcarbinol Formed by Yeast Juice.....	125

D. S. McKITTRICK. The Interrelations of Choline and Methionine in Growth and the Action of Betaine in Replacing Them	133
LETTERS TO THE EDITORS:	
GILBERTO G. VILLELA. Pteroylglutamic Acid (Folic Acid) and Neoursphenamine Toxicity.....	157
H. G. ALBAUM AND M. OGUR. An Adenine-Pentose-Pyrophosphate from Plant Tissues.....	158
THEODORE WINNICK, FELIX FRIEDBERG AND DAVID M. GREENBERG. Incorporation of C <sup>14</sup> -Labeled Glycine into Intestinal Tissue and its Inhibition by Azide.....	160
BOOK REVIEWS.....	162

# NO. 2, NOVEMBER, 1947

BARBARA A. McLAREN, ELIZABETH KELLER, D. JOHN O'DONNELL AND C. A. ELVEHJEM. The Nutrition of Rainbow Trout. I. Studies of Vitamin Requirements.....	169
BARBARA A. McLAREN, ELIZABETH KELLER, D. JOHN O'DONNELL AND C. A. ELVEHJEM. The Nutrition of Rainbow Trout. II. Further Studies with Purified Rations.....	179
WALTON E. GRUNDY, MYER FREED, HOWARD C. JOHNSON, CHARLES R. HENDERSON AND GEORGE H. BERRYMAN. The Effect of Phthalylsulfathiazole (Sulfathalidine) on the Excretion of B-Vitamins by Normal Adults.....	187
PAUL H. KOPPER AND HOWARD H. BEARD. Creatinase Activity of a Strain of <i>Pseudomonas</i> .....	195
GUSTAV J. MARTIN AND J. M. BEILER. Inhibition of the Action of Dopa Decarboxylase.....	201
LYNN DEFORREST ABBOTT, JR. Phenolsulfatase Activity.....	205
NESTOR BOHONOS, R. L. EMERSON, ALMA J. WHIFFEN, MARY PRATT NASH AND C. DEBOER. A New Antibiotic Produced by a Strain of <i>Streptomyces lavendulae</i> .....	215
WALTON B. GEIGER. Interference by Streptomycin with a Metabolic System of <i>Escherichia coli</i> .....	227
G. S. FRAPS. The Premature Death of White Rats Fed Low Levels of Carotenes from Different Sources.....	239
F. L. MUNRO AND A. M. LUPTON. The Influence of Dicumarol Treatment of Rabbits, Rats and Dogs on the Thromboplastic Activity of Their Brain Suspensions.....	245
BYRON S. MILLER AND ERIC KNEEN. The Amylase Inhibitor of Leoti Sorghum.....	251
EDWARD H. FRIEDEN AND CHESTER N. FRAZIER. The Effects of Various Agents Upon the Sensitivity of <i>Staphylococcus aureus</i> to Penicillin.....	265
GERALD OSTER, C. A. KNIGHT AND W. M. STANLEY. Electron Microscope Studies of Dahlem, Rothamsted, and Princeton Samples of Tobacco Mosaic Virus.....	279

C. A. KNIGHT AND GERALD OSTER. The Size of the Particles of Some Strains of Tobacco Mosaic Virus as Shown by the Electron Microscope. . . . .	289
F. L. MUNRO AND MURIEL PLATT MUNRO. The Preparation of Prothrombin by Adsorption on, and Elution from, Aluminum Hydroxide. . . . .	295
LAWRENCE ATKIN AND PHILIP P. GRAY. Fermentation Studies with Brewers' Yeast. I. A Synthetic Medium. . . . .	305
LETTERS TO THE EDITORS:	
GUSTAV J. MARTIN, LEO TOLMAN AND ROBERT BRENDDEL. The Hypotensive Action of 7-Methylpteroylglutamic Acid. . . . .	323
LEONORA MIRONE AND LEOPOLD R. CERECEDO. The Beneficial Effect of Xanthopterin on Lactation, and of Biotin on Reproduction and Lactation, in Mice Maintained on Highly Purified Diets. . . . .	324
S. WEISS, J. V. FIORE, AND F. F. NORD, On the Structure and Possible Functions of a Pigment of <i>Fusarium Solani</i> D <sub>2</sub> Purple . . . . .	326
BOOK REVIEWS. . . . .	329

# NO. 3, DECEMBER, 1947

FREDERICK J. DI CARLO AND SUTTON REDFERN. $\alpha$ -Amylase from <i>Bacillus subtilis</i> . I. Purification and Physical Properties. . . . .	333
FREDERICK J. DI CARLO AND SUTTON REDFERN. $\alpha$ -Amylase from <i>Bacillus subtilis</i> . II. Essential Groups. . . . .	343
BERNARD D. DAVIS. The Estimation of Small Amounts of Fatty Acid in the Presence of Polyoxyethylene Sorbitan Partial Fatty Acid Esters ("Tween") and of Serum Proteins. . . . .	351
BERNARD D. DAVIS. The Preparation and Stability of Fatty Acid-Free Polyoxyethylene Sorbitan Monooleate ("Tween" 80). . . . .	359
BENJAMIN H. ERSHOFF. Comparative Effects of Liver and Yeast on Growth and Length of Survival of the Immature Thyroid-Fed Rat. . . . .	365
C. R. BREWER, M. N. MICKELSON AND C. H. WERKMAN. Effect of Growth Substrate on the Enzymic Constitution of <i>Aerobacter indologenes</i> . . . . .	379
ELOISE JAMESON. The Determination of Plasma and Serum Protein by Electrophoresis. The Effect of Protein Concentration and Voltage Changes on Proportions of Different Proteins. . . . .	389
RALPH THEODORE HOLMAN. Crystalline Lipoxidase II. Lipoxidase Activity. . . . .	403

EUGENE F. JANSEN, ROSIE JANG AND L. R. MACDONNELL. Citrus Acetylesterase.....	415
HAROLD A. HARPER. The Microbiological Determination of Glutamine in Human Plasma.....	433
LEON A. HEPPEL, VIRGINIA T. PORTERFIELD AND EVELYN G. PEAKE. The Lipotropic Activity of Caffeine, Theobromine and Theophylline.....	439
MAURICE ERRERA AND JESSE P. GREENSTEIN. Note on the Ul- traviolet Absorption Spectra of Keto Acids and of Keto Acid Peptides.....	445
MAURICE ERRERA AND JESSE P. GREENSTEIN. Effect of Various Keto Acids on the Desamidation of Glutamine.....	449
IRENE T. GREENHUT, RICHARD L. POTTER AND C. A. ELVEHJEM. The Phenylalanine and Tryptophan Content of Meats.....	459
F. F. NORD AND JAMES C. VITUCCI. On the Mechanism of Enzyme Action. XXXI. The Mechanism of Methyl- <i>p</i> - Methoxycinnamate Formation by <i>Lentinus lepideus</i> and Its Significance in Lignification.....	465
LETTERS TO THE EDITORS:	
P. G. STANSLY AND N. H. ANANENKO. Resistance of Poly- myxin to Some Proteolytic Enzymes.....	473
BOOK REVIEWS.....	475
AUTHOR INDEX.....	481
SUBJECT INDEX.....	485
INDEX OF BOOK REVIEWS.....	492







# Purification and Some Properties of Subtilin

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Received April 15, 1947

## INTRODUCTION

Subtilin is an antibiotic substance produced by a particular strain of *Bacillus subtilis*. Its production on synthetic medium and some of its properties have already been described (1). It was shown to be a substance of low molecular weight, alkali labile, and sensitive to light. The asparagus juice medium developed in this laboratory (2) supports good growth of *B. subtilis* with the production of good yields of antibiotic activity against a number of pathogenic organisms (*in vitro*) including *Bacillus anthracis*, *Diplococcus pneumoniae*, *Neisseria gonorrhoeae*, and *Mycobacterium tuberculosis* (3).

This report deals with the method used at present for the extraction and purification of the material produced on asparagus medium by surface culturing of the organism.<sup>2</sup> The active factor has not been isolated in pure form but it has been concentrated 200- to 300-fold (dry-weight basis) from the bacterial cultures. The preparations are dull white amorphous powders which are soluble in acidified water.

## EXPERIMENTAL

*Source of Material.* The bacterial cells from which the subtilin was obtained were grown by surface culturing of *B. subtilis* on asparagus juice medium as described elsewhere (4). The surface pellicle was recovered from the culture medium by straining with cheesecloth.

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<sup>1</sup> Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

<sup>2</sup> The method as described is suitable also for recovery of subtilin from surface cultures when molasses is used as the medium (4). The yields of purified material may be somewhat lower for the molasses cultures.

**Assay Method.** Subtilin was assayed by a short incubation period turbidimetric bacteriostatic method similar to that described by McMahan (5) for penicillin. Test organisms included *Micrococcus conglomeratus*, *Staphylococcus aureus*, and *Streptococcus faecalis*. The first was used routinely, but, in general, all have given similar results. The values given in this paper are relative values based on a selected sample of partially purified subtilin (Lot L1263), the potency of which was arbitrarily designated as 100%. Potencies are expressed as per cent relative to this standard and yields as weight of 100% potency material. Details of the method are presented elsewhere (6).

**Extraction Procedure.** Sufficient 95% alcohol (ca. 2 volumes) is added to the pellicle to bring the alcohol concentration to 65–70% and the suspension is thoroughly stirred.<sup>3</sup> The cellular debris flocculates and is allowed to settle in the cold room (4°C.) overnight. Approximately two-thirds of the solution is then removed as a clear extract, while the remainder is collected by filtration.<sup>4</sup> The extraction of the subtilin by aqueous alcohol appears to be essentially quantitative, since re-extraction of the pellicle with alcohol gave only 7% more subtilin.<sup>5</sup>

**Concentration of the Aqueous Alcohol Extract.** One per cent of glacial acetic acid is added to the clear alcohol extract. The extract is concentrated *in vacuo* at about 35°C. to approximately 20% of the original volume. The concentrate is kept at 4°C. for 12–15 hours to allow the precipitate to flocculate. The precipitate is collected by centrifugation and washed twice with two volumes of water. The supernatant liquor and the wash waters are discarded. The washed precipitates are lyophilized, yielding a dark brown finely divided powder (Fraction A). Approximately 50 g. are obtained from 100 l. of original culture, although some variation occurs in individual experiments.

**Extraction of Fraction A with 95% Ethyl Alcohol.** Subtilin is essentially insoluble in 95% ethyl alcohol while approximately  $\frac{2}{3}$  of the solids of Fraction A are soluble. The dried material is, therefore, extracted with 95% alcohol 4 times using 15 ml. of alcohol/g. of solids for each extraction. The insoluble material is collected after each

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<sup>3</sup> The harvested pellicle, consisting of bacterial cells and residual medium, contained about 10% solids on a volume basis.

<sup>4</sup> A basket centrifuge fitted with a solid head and skimmer was used.

<sup>5</sup> Subtilin may be extracted from surface or submerged (7) whole cultures with  $\frac{1}{2}$  volume of *n*-butyl alcohol after adjustment of the pH to 2.0–2.5 by adding 6 *N* hydrochloric acid. The two phases are thoroughly homogenized by suitable means for two hours and then separated by means of a Sharples centrifuge. The distribution coefficients are such that approximately 85–90% of the subtilin is extracted by the water-saturated butyl alcohol. The butyl alcohol extract is concentrated *in vacuo* (35–40°C.) adding water continuously in order to permit evaporation of the low-boiling binary. After the volume has been reduced to 12–15% of the original no more water is added and evaporation is continued until the butyl alcohol is free of water and the solute precipitates. The precipitate is collected by centrifugation. Subsequent purification has been the same as for the ethyl alcohol extraction method. The products of the two methods appear to be similar, but the yield is lower with the butyl extraction procedure due to greater losses in the purification steps. Possible changes in this procedure to increase the yield are being studied.

extraction by centrifugation and is finally lyophilized (Fraction B). The 95% alcohol extracts are discarded since they contain only small amounts of activity.

At this stage of purification the recovery of antibiotic has been found to be essentially quantitative and the activity of the solids had been increased 20–50 times (Table I). The solids present in Fraction B are largely insoluble in water except at

TABLE I  
*Purification of Subtilin by Alcohol Extraction*

Prep. No.	Alcohol extract		Fraction A <sup>1</sup>			Fraction B <sup>2</sup>		
	Dry wt.	Subtilin <sup>3</sup>	Dry wt.	Subtilin <sup>3</sup>	Recovery	Dry wt.	Subtilin <sup>3</sup>	Recovery
	g.	g.	g.	g.	per cent	g.	g.	per cent
SP2B	316	3.28	23.3	3.66	111	5.75	3.62	110
SP2C	—	2.78	—	—	—	4.60	3.20	108
SP2F	289	4.58	27.4	4.56	100	5.62	4.00	88
SP6A	145	7.12	37.6	5.45	77	4.59	5.12	72
SP7A	322	12.4	36.8	12.4	100	14.6	12.0	97
SP7B	315	11.7	37.7	12.6	108	14.8	11.6	99

<sup>1</sup> Filter cake.

<sup>2</sup> Alcohol-extracted filter cake.

<sup>3</sup> Standard equivalents by *Staphylococcus aureus* assay.

very acid pH. The active material is also largely insoluble in water, due apparently to the presence of a dark gummy material to which the antibiotic adheres tenaciously. Difficulty was encountered in separating this material from the active fraction. Active fractions essentially free of the gummy material were obtained by emulsifying Fraction B in 1% acetic acid solution and dialyzing the suspension against 1% acetic acid. Subtilin diffused through cellophane membranes fairly readily and, on drying the dialyzate, a dull white powder was obtained which dissolved readily in water. This preparation was somewhat more active than the starting material. The recovery, however, was low and the process tedious.

Fairly good separation was also obtained by dissolving Fraction B in glacial acetic acid and diluting with 5–10 volumes of water. Most of the gum precipitated, leaving appreciable amounts of subtilin in solution, from which it was recovered by lyophilization. The product was, however, not satisfactory from the standpoint of solubility and the yield was low.

*Extraction with 85% Ethyl Alcohol Containing 1% Acetic Acid and 1% NaCl.* The procedure finally adopted for further purification of Fraction B is based on the observation that in acidified alcoholic solutions the presence of sodium chloride had a marked effect on the solubility of the impurities and of the active material (Table II). In 85% alcohol containing 1% acetic acid the solids of Fraction B are still soluble to an appreciable extent but there is no effective differential solubility of the gummy fraction and the antibiotic. When 1% NaCl is added, however, it has a "salting in" effect on the major impurities, increasing the solubility by more than 70% while

TABLE II

*Effect of NaCl on Solubility of Fraction B and of Subtilin in Aqueous Acidified (1% CH<sub>3</sub>COOH) Ethyl Alcohol*

Alcohol conc.	Solubility Mg./ml.		Solubility Mg. activity <sup>1</sup> /ml.	
	No NaCl	2% NaCl	No NaCl	2% NaCl
<i>per cent</i>				
25	2.4	1.2	2.2	0.45
40	3.4	4.6	2.4	3.5
55	6.4	19.0	4.0	16.8
70	10.6	19.2	5.6	13.3
85	7.2	12.2 <sup>2</sup>	4.4	<2.0 <sup>2</sup>

<sup>1</sup> Assayed with *Micrococcus conglomeratus*.

<sup>2</sup> 1% NaCl (approximately saturated).

simultaneously the solubility of subtilin is reduced 2-fold or more. It is, therefore, possible to make an effective separation.

Fraction B is extracted at room temperature 4 times with 100-ml. portions of the mixed solvent/5 g. of solids. The insoluble material (Fraction C) is collected by centrifugation. The loss of active material in the extracts was usually 15–20% of the total (Table III).<sup>4</sup>

*Fractionation with Acetate Buffer.* The residue from the 85% alcohol-1% salt extraction is further purified by fractionation in a 0.08 *M* acetate buffer, pH 4.5. To obtain as good dispersion in the solution as possible, Fraction C is first dissolved with stirring in 0.08 *M* aqueous acetic acid (35 ml./g. material). The solution is then adjusted to pH 4.6–4.7 by the careful addition of *N* KOH with stirring. A brown material which still contains much of the active substance flocculates out. The precipitate is centrifuged off and again extracted as before and the process repeated 4 more times. The maximum solubility of the active substance in this buffer appears to be 5–6 mg./ml.; consequently, repeated extractions with this volume are essential to recover most of the active substance. Essentially the same result may, however, be achieved by one extraction with 6 times the amount of acetate. The brown residue is essentially insoluble and hence is eliminated. Approximately 10–15% of the subtilin is lost in the acetate-insoluble residue which is discarded.

Difficulty was encountered in obtaining clear solutions when removing the brown gummy precipitate from the acetate extract by centrifugation. This was overcome by

<sup>4</sup> Approximately 50% of the active material may be recovered from the 85% alcoholic solution by drying and reworking the solute. The dry material is dissolved in glacial acetic acid and the insoluble sodium chloride removed by centrifugation. The acetic acid is removed by low temperature sublimation, and the residue extracted with saline acidified 85% alcohol as before.

TABLE III

*Purification of Subtilin with 85% EtOH Containing 1% NaCl and 1% CH<sub>3</sub>COOH and with Acetate Buffer*

Experi- ment No.	Acetate buffer							
	Fraction B		Fraction C <sup>1</sup>		Recovery	Fraction D <sup>2</sup>		Recovery
	Solids	Subtilin <sup>3</sup>	Solids	Subtilin <sup>3</sup>		Solids	Subtilin <sup>3</sup>	
	<i>g.</i>	<i>g.</i>	<i>g.</i>	<i>g.</i>	<i>per cent</i> <sup>4</sup>	<i>g.</i>	<i>g.</i>	<i>per cent</i> <sup>4</sup>
83	25.5	19.3	18.2	17.0	88	10.0	18.6	96
86	26.6	21.0	18.9	18.0	87	8.4	14.2	68
87	26.6	21.0	17.8	19.0	86	7.8	13.2	63
73	5.0	5.4	2.5	3.9	72	1.6	3.5	65
75	39.0	40.0	20.6	27.7	69	11.3	21.2	53
77	21.3	22.7	13.2	19.2	85	8.6	16.3	70
76	18.8	24.0	13.3	20.4	85	—	—	—

<sup>1</sup> 85% EtOH, 1% NaCl, 1% CH<sub>3</sub>COOH-insoluble.

<sup>2</sup> 0.16 M acetate-soluble.

<sup>3</sup> Standard Equivalents by *Micrococcus conglomeratus* assay.

<sup>4</sup> Based on original alcohol extract.

treating the centrifuged acetate solution with 0.3% "Hyflo" filter aid (Johns Manville Corp., New York, N. Y.) and filtering through a "Sparkler" filter (Sparkler Mfg. Co., Mundelcin, Ill.). The solutions thus obtained were clear and no loss of subtilin has been observed. Limited toxicological data indicates that this step in the procedure lowers the toxicity of the final preparations very markedly.

The acetate extracts are freed from cations by passage through a suitable cation exchange resin (IR 100) and subsequently the pH is brought to 3.2 by stirring with an anion adsorbent (IR 4). This procedure is necessary in order that the product be freed of salt and essentially free of the chloride ion. The presence of salt reduces the solubility of the active material tremendously.

The extract is concentrated to approximately one-fourth of its volume *in vacuo* below 35°C. and then lyophilized. The product (Fraction D) is a dull white powder which dissolves in water to the extent of 10–20% (Table III).

An unexpected effect was observed when HCl was substituted for acetic acid in the preparation of subtilin solutions. They are regularly acidified with acetic acid (to below pH 5.0) to assure a stable soluble product. When HCl was used for the same purpose all activity was lost during drying from the frozen state. Consequently, it is essential that the chloride ion be removed as far as possible from the acid solutions before drying.

*Properties of Subtilin.* The most soluble preparations of subtilin (those prepared by dialysis) are soluble to at least 30% in salt-free

acidified solution. If the pH of the solution is adjusted to 7.0, the solubility is reduced to less than 0.5% and at pH 8.5 the solubility is still lower. Subtilin apparently is a basic substance whose acid salts are soluble.

The solubility of subtilin in acid solution is markedly decreased by the presence of salt. In the presence of 0.5–1% sodium chloride the solubility is reduced to approximately 0.5–0.2%. This fact has been a source of difficulty in the purification procedure, as it is essential that all preparations be salt free. Since subtilin diffuses through membranes, dialysis cannot be used for this purpose. Ion exchange resins are therefore used.

The subtilin preparations are essentially insoluble in all dry organic solvents such as ethyl alcohol (above 95%), butyl alcohol, acetone,

TABLE IV  
*pH Stability of Subtilin*

Expt. A. Subtilin prepared by adsorption and dialysis. 0.05% solutions exposed to room temperature for 20 hrs.

pH	Buffer	Activity <sup>1</sup> retained Per cent
4.5	0.1 M acetate	98
5.5	0.1 M acetate	98
6.5	0.1 M phosphate	94
7.5	0.1 M phosphate	86
8.5	0.1 M borate	84
9.0	0.1 M. borate	65
9.6	0.1 M glycine	30
10.0	0.1 M glycine	13
11.2	0.1 M glycine	4

Expt. B. Subtilin (reference standard) in 0.1% solution incubated at 37°C. for 1 and 6 days in mixed buffer 0.05 M with respect to phosphate, acetate, borate, and KCl.<sup>2</sup>

pH	Activity <sup>1</sup> retained after:	
	1 day Per cent	6 days Per cent
6.8	65	43
7.3	64	32
7.7	58	21
8.2	46	12
8.6	27	6
9.0	15	5

<sup>1</sup> Assayed with *Staphylococcus aureus*.

<sup>2</sup> pH adjusted with 0.1 N KOH.

ether, petroleum ether, chloroform, and amyl alcohol. In *n*-butyl alcohol saturated with water they were soluble to approximately 0.5%, while in aqueous ethyl alcohol they were soluble to the extent of at least 10% at alcohol concentrations below 80%. They were also readily soluble in glacial acetic acid or in aqueous solutions of acetic acid.

The subtilin preparations are relatively stable at acid pH (2.5 or above) but were decreasingly stable with increasing pH above pH 7.0 (Table IV). Dilute solutions (40  $\gamma$ /ml.) of subtilin in pH 2.5 HCl were stable for long periods when stored in the refrigerator (Table V). Dry

TABLE V  
*Effect of Storage Conditions on the Stability of Subtilin*

Preparation	Potency relative to standard L1263	Storage conditions	Relative potency
	<i>Per cent</i> (100)		<i>Per cent</i> (100)
Standard, lot L1263		solid; in refrigerator <sup>1</sup> solid; 10 months at 25°C.	93 ( <i>M. conglomeratus</i> ) 84 ( <i>S. faecalis</i> ) 97 ( <i>S. aureus</i> )
		solid; 10 months at 35°C.	77 ( <i>M. conglomeratus</i> ) 68 ( <i>S. faecalis</i> ) 78 ( <i>S. aureus</i> )
		solution (40 $\gamma$ /ml. in pH 2.5 HCl); 8 months in refrigerator	97 ( <i>M. conglomeratus</i> ) 98 ( <i>S. faecalis</i> ) 102 ( <i>S. aureus</i> )
Partially purified subtilin, lot L1277	152 ( <i>M. conglomeratus</i> ) 124 ( <i>S. faecalis</i> ) 147 ( <i>S. aureus</i> )	solid; in refrigerator <sup>1</sup>	(100)
		solid; 8 months at 25°C.	82 ( <i>M. conglomeratus</i> ) 73 ( <i>S. faecalis</i> ) 80 ( <i>S. aureus</i> )
Partially purified subtilin, lot 1242	101 ( <i>M. conglomeratus</i> ) 121 ( <i>S. aureus</i> )	solution (40 $\gamma$ /ml. in pH 2.5 HCl); in refrigerator <sup>1</sup> solid; 5 months at 25°C.	(100) 59 ( <i>M. conglomeratus</i> ) 66 ( <i>S. aureus</i> )
Lyophilized whole <i>Bacillus subtilis</i> culture (grown on asparagus juice)	0.73 ( <i>M. conglomeratus</i> )	solid; in refrigerator <sup>1</sup>	(100)
		solid; 7 months at 25°C.	30 ( <i>M. conglomeratus</i> )
Partially purified subtilin, lot L1254	25-50 ( <i>S. aureus</i> )	solution (40 $\gamma$ /ml. in pH 2.5 HCl); in refrigerator <sup>1</sup> solution (1000 $\gamma$ /ml. in 1 <i>N</i> HCl); 2 days in refrigerator the same; 30 days in refrigerator	(100) 81 ( <i>S. aureus</i> ) 15* ( <i>S. aureus</i> )

<sup>1</sup> Complete stability was assumed for this control storage condition; parallel assays for all storage conditions were made after any particular period of storage.

preparations lost appreciable activity over a period of months on storage at 25 or 35°C. as compared with storage in the refrigerator. Subtilin in *N* HCl solution lost activity fairly rapidly, even in the cold.

The activity of subtilin is decreased in the presence of crystallized pepsin and trypsin. Table VI presents the results obtained from an



TABLE VI  
*Effect of Crystalline Pepsin and Trypsin on Subtilin<sup>1</sup>*

Description of sample	Buffer	Retention of activity Per cent
Control	pH 2.0 HCl	88
Pepsin treated	pH 2.0 HCl	29
Control	pH 7.3, 0.1 M phosphate	70
Trypsin treated	pH 7.3, 0.1 M phosphate	13

<sup>1</sup> Subtilin (0.1%) plus enzyme (0.02%) incubated at 37°C. for 24 hours.

experiment in which the effects of incubation with crystalline pepsin and trypsin were determined.

The specific rotation of several subtilin preparations has been determined with 2.5% solutions of the preparations in 1% acetic acid. The material was levorotatory, the specific rotation  $[\alpha_D^{23}]$  of 6 preparations varying from 29.0–35.0.

15.8% nitrogen (moisture free basis) was found in subtilin preparations by Kjeldahl analysis. Approximately 11% of the nitrogen was present as amino nitrogen. After acid hydrolysis 80% of the nitrogen was present as amino nitrogen. Only a trace of phosphorus was found, but sulfur was present to the extent of 4.2%.

## DISCUSSION

Several reports have appeared recently concerning antibiotics which resemble subtilin in being produced by strains of *B. subtilis* or in other respects. Properties bearing on the identity or non-identity of these substances with subtilin will be reviewed briefly.

Bacitracin (8), produced by a bacterium of the *B. subtilis* group, like subtilin, is stable in acid but labile in alkaline solution, and is extractable with butanol but not with ether. It is described as neutral, whereas subtilin is definitely basic, and it "resists digestion with pepsin or trypsin" whereas subtilin activity is decreased by these enzymes.

Comparative antibiotic spectra differentiate subtilin from bacillin, produced by a strain of *B. subtilis*, and simplexin, produced by a strain of *B. simplex* (9). Like subtilin, bacillin is not extracted with ether, but, unlike subtilin, bacillin is soluble in ethanol containing 5% water.

Eumycin (10), produced by certain strains of *B. subtilis*, also resembles subtilin in being an acid-stable, alkali-labile, butanol and ethanol-soluble, ether-insoluble material. Unlike subtilin, but like tyrothricin,

it is precipitated by acidifying the cultures. It is characterized by high fungistatic activity and relatively little inhibition of *Staphylococci*.

A strain of *B. licheniformis* (closely related to *B. subtilis*) produces an antibiotic against *M. tuberculosis* which differs from subtilin in that the activity of cultures is precipitated rather than extracted by 70–80% ethanol (11). It has also been reported (12) that *B. subtilis* produces active material which is effective against *M. tuberculosis*. This substance is extractable from acid medium with ether or chloroform and is soluble in aqueous solutions at pH 8.5.

Ramon and co-workers (13) have demonstrated that culture filtrates from certain strains of *B. subtilis* have the ability to inactivate certain toxins as well as to inhibit growth of the pathogenic microorganisms which produced them. The active factor is stable both to heat and to formaldehyde, and is named "subtiline."

Colistatin (14), an antibiotic produced by an apparently unidentified aerobic sporulating bacillus, appears to be differentiated from subtilin by the fact that colistatin is not extractable from the culture liquid by butanol as is subtilin. Also, charcoal does not adsorb colistatin from acid solution, while subtilin is readily adsorbed.

Diplococcin, produced by certain *Streptococci* (15), resembles subtilin in its probable polypeptidic nature, its insolubility in absolute ethanol, its alkali lability, and in being laevo rotatory. Unlike subtilin, it lacks sulfur. Final demonstration of identity or non-identity of any of these factors with subtilin will necessarily depend on the isolation of the pure substances.

The behavior of subtilin in the presence of salt is perhaps its most outstanding characteristic. In salt-free acidified aqueous solution subtilin is soluble to the extent of 10–20%. The salting-out effect of NaCl is very great, 1% NaCl reducing the solubility to 0.2–0.3%. This effect is not specific for the active material, since the associated impurities behave in a similar manner. Due to this fact, it is difficult to accomplish any marked purification in aqueous solution, since the opportunity for variable conditions is limited. The situation in aqueous alcoholic solutions, however, is more favorable for fractionation. In alcoholic solutions below 50% alcohol, sodium chloride also has a salting-out effect, although this effect is less than in water. Between 50 and 75% alcohol concentration, the solubility of subtilin is increased by the presence of 2% NaCl. The effect is again reversed at 85%

alcohol and subtilin is salted out by 1% NaCl. As already related, the differential effect of NaCl on solubility of subtilin and of impurities resulted in an appreciable purification. In solutions of this type the effects of several variables can be balanced against each other and conditions favorable for purification be selected.

### SUMMARY

Subtilin, an antibiotic material bacteriostatic against *Micrococcus conglomeratus*, *Staphylococcus aureus*, and *Streptococcus faecalis* as assay organisms, has been purified from pellicles produced by a particular strain of *Bacillus subtilis* on asparagus butt juice medium. The subtilin is extracted from the pellicle with 70% ethanol. The extracts are concentrated *in vacuo* to remove ethanol, whereupon the active material precipitates. The dried filter cake is extracted successively with 95% ethanol, and with 85% ethanol containing 1% each of acetic acid and NaCl to remove inactive contaminants. The active material is then obtained in solution by extracting the insoluble residue with 0.16 *M* acetate at pH 4.6. The extracts are treated with Hy-Flo, deionized with exchange resins, concentrated, and lyophilized to yield a dull white powder.

The subtilin preparations are soluble to more than 10% in salt-free acidified water, but less than 0.5% at pH 6-9. In the presence of 0.5% NaCl at acid pH the solubility is less than 0.5%. The preparations are soluble in 0-80% ethanol, and in methanol, but not in dry ethanol, butanol, amyl alcohol, acetone, ether, petroleum ether, or chloroform. Subtilin is about 0.5% soluble in *n*-butanol saturated with water.

Subtilin is inactivated by alkali and the activity is decreased by incubation with pepsin and trypsin. It diffuses fairly rapidly through cellophane. The preparations contain 15.8% total (Kjeldahl) nitrogen. The amino nitrogen content of 1.6% increases to about 11% after acid hydrolysis. They contain 4.2% sulfur and a trace of phosphorus and are laevorotatory.

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# Zinc as an Essential Element for Growth and Subtilin Formation by *Bacillus Subtilis*<sup>1</sup>

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Received May 1, 1947

## INTRODUCTION

The element zinc has apparently never been demonstrated as a nutritional requirement of a bacterium. Studies of the nutritional factors conducive to subtilin production by *Bacillus subtilis* indicated that zinc was nutritionally essential for *B. subtilis* (Feeney *et al.* (3)). Experiments designed to prove a requirement for zinc by *B. subtilis* are reported in this paper. General cultural conditions for the production of subtilin have been recently described (Lewis *et al.* (6)).

## PROCEDURE

The culture of *B. subtilis* employed in these investigations was the non-adherent stock culture previously described (Lewis, *et al.* (6)). It was carried on agar slants of Medium II of Schmidt and Moyer (13) and stored in a refrigerator between transfers. For use, transfers were made from such stock slants into 250-ml. Erlenmeyer flasks containing 50 ml. of the "zinc-free" medium described below. Very slight growth was obtained on this medium after 24-48 hours' incubation at 35°C. The pellicles on these cultures were so light and fragile that a finely dispersed inoculum could be obtained by simply swirling the flasks. Five-tenths ml. of inoculum was pipetted into each flask of experimental medium.

All glass vessels employed were Pyrex, and all equipment was cleaned with dichromate cleaning solution and well rinsed with distilled water prior to use. Extreme precautions were taken to prevent contamination of media and reagents with minerals. Pipettes were sterilized unwrapped in Pyrex glass cases. Double distilled water was used throughout.

The base medium had the following composition: sucrose, 100 g.; asparagin, 2.0 g.; glutamic acid, 2.0 g.; tryptophan, 0.10 g.; Na<sub>2</sub>SO<sub>4</sub>, 4.0 g.; (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 8.0 g.; NaCl,

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<sup>1</sup> Published in abstract form in the *Federation Proc.* May 18-22 (1946).

\* Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

0.30 g.; a supplementary salt mixture; 100 ml. of an extract<sup>2</sup> prepared from Steffen's waste liquor concentrate; and double-distilled water to give 1 l. of medium. The supplementary salt mixture gave the following salts in the amounts indicated/l. of medium:  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.51 g.;  $\text{CaCl}_2$ , 0.14 g.;  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.019 g.;  $\text{KCl}$ , 0.095 g.; and  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.18 g. This medium and other nutritional observations will be described in a subsequent publication (Feeney *et al.* (3)). It has not been employed for routine antibiotic production.

Removal of zinc from the medium was accomplished by extractions with carbon tetrachloride solutions of dithizone (diphenylthiocarbazone). In the preparation of a typical lot of medium the following procedure was employed: 750 ml. of double-strength medium (with the supplementary salt mixture omitted) was adjusted to pH 8.5 with dilute  $\text{NaOH}$ , extracted with 300 ml. of a 0.01% solution of dithizone in carbon tetrachloride, and then re-extracted with 50 ml. more of the dithizone solution. The excess dithizone and carbon tetrachloride remaining in the medium were removed by several extractions with chloroform. The medium was finally adjusted to pH 6.9–7.0 with  $\text{HCl}$ . Semiquantitative analyses (Cowing and Miller (2)) of this purified medium showed it to contain  $<0.004$  p.p.m. zinc. The reagents for the above treatment and the double-distilled water were tested and found "free" of zinc.

The above medium was dispensed in 25-ml. amounts in 250-ml. Erlenmeyer flasks. The omitted salt mixture was now added to the flasks, and zinc as  $\text{ZnCl}_2$  was added in graded amounts to the flasks. The volume of medium in each flask was then made to 50 ml. by adding double-distilled water. The flasks were plugged with cotton, sterilized at  $120^\circ\text{C}$ . and 15 pounds pressure for 15 minutes, inoculated, and incubated at  $35^\circ\text{C}$ .

The antibiotic assays were performed according to Lewis *et al.* (7) with *Micrococcus conglomeratus* as the test organism. Antibiotic yields were expressed in mg./l. of medium as determined against the arbitrary subtilin standard (L1263) used in this laboratory. Dry weights of pellicles were obtained by filtering the blended cultures through Seitz filter disks and washing, drying at  $55^\circ\text{--}60^\circ\text{C}$ . for 24–48 hours, and weighing the residues.

## RESULTS

The data of Table I demonstrate that the zinc requirement was similar for maximum growth and maximum antibiotic formation. However, in the absence of added zinc, slight growth was obtained with no detectible antibiotic formation. In a number of other experiments with young cultures ( $<48$  hours old) the parallelism between the requirement for growth (estimated visually) and antibiotic yield appeared less close than that illustrated in Table I. Flasks supplied with sufficient zinc to give optimal growth might contain only 20–30%

<sup>2</sup> This extract was prepared by butanol extraction of acid-hydrolyzed Steffen's waste-liquor concentrate (O'Day and Bartow (11)). One ml. of the extract was equivalent to 0.5 g. of the concentrate. The Steffen's waste-liquor concentrate was kindly supplied by the International Minerals and Chemicals Corporation.

TABLE I

*Antibiotic and Pellicle Yields in the Presence of Graded Amounts of Zinc Added to a "Zinc-Free" Medium<sup>1</sup>*

Amount of zinc <sup>2</sup> added p.p.m.	Dry weight of pellicle <sup>3</sup> mg.	Antibiotic yield <sup>3</sup> mg./l.
0.00	33	<16
0.03	58	30
0.10	130	120
0.25	160	520
0.40	240	720
0.60	230	690
0.80	220	670
1.00	240	610
10.0	220	620

<sup>1</sup> The "zinc-free" medium is described in the text.

<sup>2</sup> Zinc was added as  $\text{ZnCl}_2$ .

<sup>3</sup> The contents of duplicate flasks were blended together after 64 hours of incubation. One-half (50 ml.) of each combined and blended sample was employed for antibiotic assay. The other half was filtered through a bacterial filter and the dry weight of the residue was determined.

of the amount of antibiotic contained in flasks with more zinc. In addition to reductions in pellicle weights in the presence of suboptimal amounts of zinc, there were also differences noted in the physical characteristics of the pellicles. In the presence of approximately 50% of the amount of zinc necessary for maximum growth, growth was not only reduced but atypical. Pellicles were wet, sometimes submerged, and without the characteristic wrinkling found in cultures adequately supplied with zinc.

The effect of time of incubation on the magnitude of the zinc requirement is shown in Fig. 1. The minimum requirement decreased from approximately 1.1 p.p.m. with the 50-hour period of incubation to 0.6 p.p.m. with the 88-hour period. In other experiments (see Table I) the minimum requirement was as low as 0.4 p.p.m. This variation in requirement appeared to be dependent, not only upon the time of incubation but also upon uncontrollable variations in the time required to attain maximum growth in each experiment.

In a limited number of experiments no other element or combinations of elements were found which could adequately substitute for zinc. A partial substitution was found only with cadmium, the element most closely related chemically to zinc. Cadmium, tested at 0.2 and



1.0 p.p.m., gave an antibiotic yield of 110 mg./l. for both concentrations. The antibiotic yields in this experiment were <20 mg./l. in the absence of added zinc and >600 mg./l. in the presence of 0.4–10.0 p.p.m. added zinc. The elements which showed insignificant responses were: Al, Sr, Ga, Ba, Sn, Ni, Tl, Pb, Cu, Co, Mo, Cr, and Hg.

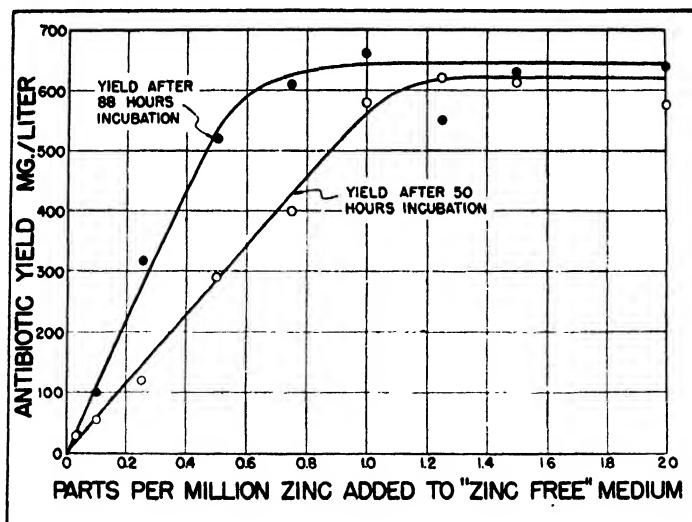


FIG. 1. The effect of zinc on antibiotic yield. Zinc was added as  $\text{ZnCl}_2$ . The "zinc-free" medium is described in the text. Antibiotic yields in the absence of zinc or with 0.01 p.p.m. of added zinc were <20 mg./l. In this experiment duplicate sets of flasks were harvested at the times indicated.

## DISCUSSION

The essentiality of zinc in the nutrition of plants and lower fungi has been well established (Foster (4); Arnon (1); Stiles (14)). Although zinc has long been incorporated in bacteriological media, and numerous reports have indicated slight, or vague nutritional effects with zinc, the essentiality of zinc for a bacterium does not appear to have been previously established. Mueller (9), in nutritional studies with the diphtheria bacillus, presented data which appears the most conclusive evidence in the literature for the stimulation of bacterial growth by zinc. However, Mueller (10) pointed out that these results should be regarded as inconclusive. Among the many other reports in which

beneficial effects by zinc were noted are those of Lipman and Burgess (8), Perlman (12), Stockton and Wyss (15), and Hutner (5).

In our investigation, a partial zinc deficiency was first observed on an unpurified medium. The heavy growth obtainable with this strain of *B. subtilis* probably resulted in a more easily demonstrable zinc requirement than might be the case with other bacteria producing smaller cell yields.

#### ACKNOWLEDGMENTS

The authors wish to express their appreciation to E. M. Humphreys and P. A. Thompson for technical assistance, and to J. C. Lewis and C. M. Johnson for valuable suggestions regarding bioassay and mineral analyses.

#### SUMMARY

Zinc was found to be essential for growth and subtilin formation by *Bacillus subtilis* in shallow layer stationary cultures. The minimum requirement for zinc was approximately 1.0 p.p.m. Cadmium, which only partially substituted for zinc, was the only element of those tested capable of substitution.

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# Essential Fatty Acid Metabolism. I. Essential Fatty Acid Content of Rats on Fat-Free and Pyridoxine-Free Diets\*

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Received October 11, 1946; Revised July 7, 1947

## INTRODUCTION

A deficiency disease in rats caused by the exclusion of fat from the diet was announced in 1929 by Burr and Burr (1). The chief symptoms of this syndrome were scaly skin, scaly tail, cessation of growth and death. The unsaturated fatty acids, linoleic (2), linolenic (2), arachidonic (3), all polyunsaturated fatty acids, were reported capable of preventing and curing this disease. Burr characterized them as the essential fatty acids. Later he concluded that only linoleic acid is indispensable (19).

Another deficiency disease has symptoms closely resembling these (4). György (5) in 1934 described a dermatitis produced by the withdrawal from the diet of one of the B vitamins, which later was identified as pyridoxine (6). Among the symptoms of this disease are scaliness of the skin, dandruff, cessation of growth, and death.

That these two deficiencies had symptoms in common was noted by Hogan and Richardson (7). Quackenbush and his coworkers studied an acute acrodermatitis caused by the withdrawal of both fat and pyridoxine (8). Since pyridoxine and the complex of water soluble factors used by these investigators materially alleviated the disease and ethyl linoleate alone afforded even greater relief, a relationship between the essential fatty acids and pyridoxine was proposed but not elucidated.

From other sources there is evidence that pyridoxine is concerned with fat metabolism, but considerable confusion exists as to its role. Birch and György in 1936 (9) suggested that fat had a sparing action on the need for pyridoxine. Birch (10) considered that the physiological function of B<sub>6</sub> was in connection with the utilization of the unsaturated fatty acids. Engel (11) has stated that pyridoxine with pantothenic acid increases the quantity of fat synthesized by the rat. McHenry and Gavin (12) concluded from their experiments that pyridoxine is essential for the synthesis of fat from protein in the mammalian body. More recent work from Umbreit and Gunsalus

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\* Presented in part before the Am. Chem. Soc., September, 1946.

(13) has shown that pyridoxine is concerned in the decarboxylation of amino acids. This may be the necessary step in the production of fat from protein.

No suggestion has been made of a mechanism by which pyridoxine could be involved in the metabolism of the essential fatty acids.

To elucidate some of these questions, a series of studies is contemplated in this laboratory. The present paper deals with the relationship of growth rates, deficiency symptoms and analyses of fats in rats on otherwise complete diets, but having the double deficiency produced by the lack of both essential fatty acids and pyridoxine. A further comparison is made of the fat composition of rats relieved of the symptoms by each essential administered alone.

Inasmuch as pyridoxine also had been shown to be concerned in the normal metabolism of tryptophan (14), this amino acid was in some cases used as an additional supplement, but since it was not found to influence the factors studied, detailed reference to it will be omitted.

## METHODS

### *Animals*

The animals employed in this investigation were male albino rats from the Lankenau Hospital Research Institute colony.

### *Diets*

Two diets were used, the first of which, diet A, was fed to lactating mothers and was restricted in pyridoxine and fat to partially deplete the young of these two essential supplements. Diet A consisted of casein 8.4%, potato meal 87.7%, Osborne and Mendel salts 1.6%, agar 2%, cystine 0.2%, and choline hydrochloride 0.1%. Water-soluble vitamins were administered by pipette in dextrose solution. The daily dose consisted of thiamine hydrochloride 40  $\gamma$ , calcium pantothenate 200  $\gamma$ , *p*-aminobenzoic acid, nicotinic acid and inositol 500  $\gamma$  each, biotin methyl ester 0.25  $\gamma$ . The fat-soluble vitamins were fed by pipette daily and consisted of 0.125 mg. crystalline carotene, 0.05 mg. of 2-methyl-4-naphthoquinone, 0.05 mg. of  $\alpha$ -tocopherol and 25 U.S.P. units of viosterol dissolved in 0.1 ml. of ethyl laurate.

The second diet, B, was used for all rats after weaning. It contained casein 20%, cerelese 72.4%, salts 4%, agar 3%, cystine 0.4%, and choline hydrochloride 0.2%. The water-soluble vitamins were fed daily and the fat-soluble vitamins administered three times a week in the same doses as was given daily in diet A.

Pyridoxine, when given as a supplement, was added to the water-soluble vitamin solution at 50  $\gamma$  daily. When tryptophan was employed, 50 mg. were mixed with the daily portion of the diet.

Ethyl linoleate was prepared from tetrabromostearic acid as described in Organic Syntheses (15). We are indebted to Dr. Weinhouse for this preparation. It was administered to the rats by pipette at the level of 30 mg. a day.

### Methods of Analyses

*Extraction of Fats.* The animals were killed with chloroform and the intestinal tracts removed and washed. The entire carcass, including the intestinal tract, was ground and covered with alcoholic potassium hydroxide. After a few days the tissues were in solution and were refluxed for complete saponification. They were then extracted with petroleum ether to separate the non-saponifiable lipids. The residues were acidified and re-extracted with petroleum ether to isolate the fatty acids.

*Iodine Values.* Iodine values (I.V.) were obtained by the method of Wijs (16).

*Spectroscopic Analyses of Unsaturated Fatty Acids.* These analyses were made by measuring the ultraviolet absorption of the soaps of the extracted fatty acids. The isomerization technique described by Mitchell, Kraybill and Zscheile (17) was employed. The specific absorption coefficients of the soaps of linoleic and linolenic acids reported by Beadle and Kraybill (18) were checked by Dr. Sidney Weinhouse and his staff, who performed these analyses. Since the specific absorption constants of linoleic and linolenic acids agreed with those reported, the specific absorption constant for arachidonic acid as given by Beadle and Kraybill (18), along with those of linoleic and linolenic acids, was used in calculating the percentage of these soaps from the absorption of the mixed extracted fatty acid soaps. These values, together with the iodine values of the acids, were in turn used in computing the percentage of oleic acid present in the fatty acids of the rats.

$$\begin{aligned}\% \text{ oleic acid} &= \text{I.V. of mixed acids} - (\text{I.V. of linoleic acid} \times \% \text{ linoleic acid}) \\ &\quad - (\text{I.V. of linolenic acid} \times \% \text{ linolenic acid}) \\ &\quad - (\text{I.V. arachidonic acid} \times \% \text{ arachidonic acid}) / \text{I.V. of oleic acid.}\end{aligned}$$

It should be pointed out that the calculations are only tentative, since the constants given for the pure acids may be modified by the presence of other acids.

### OUTLINE OF EXPERIMENT

The double deficiency disease caused by the withdrawal of both pyridoxine and linoleic acid manifested itself after about 8 days on the restricted diet (B) by inflammation between the digits of the front feet. Scaliness of the forefeet followed. This was called the initial stage of the disease. Rat No. 1 was killed for analysis at this time (Table I).

Gradually the front feet, and later the rear feet, became swollen and denuded. The acrodermia symptoms of edema, swelling and crustation around the nose and lips, with thinning of the hair in these parts and under the chin appeared. The tail was sometimes scaly. Priapism was present in all rats except No. 5. This constituted the advanced stage and was characteristic of the animals at about the fifth week of deprivation. Rat No. 2 was killed and analyzed at this time.

With rat No. 3, kept until the terminal stage, the symptoms became

more severe. It assumed a typical haunched stance, its paws and snout were swollen and denuded, and the animal was obviously very sick. In spite of the aggravated condition of its symptoms it was able to withstand the double deficiency for 39 more days, at which time it also was killed for analysis.

TABLE I

*Age and Weights of Rats on Fat- and Pyridoxine-Free Diets,  
and After Refeeding with Various Supplements*

All were 30 days of age at the start.

Rat No.	Supplement	Deficiency period					Supplemented period		
		<sup>a</sup> Duration	<sup>b</sup> Wt. at weaning	<sup>c</sup> Max. wt.	<sup>d</sup> Final wt.	$\frac{c-d}{c-b} \times 100$	<sup>f</sup> Duration	<sup>g</sup> Final wt.	$\frac{g-d}{d} \times 100$
		<i>days</i>	<i>g.</i>	<i>g.</i>	<i>g.</i>		<i>days</i>	<i>g.</i>	
1		20	51	74	51	100			
2		38	52	71	63	42			
3		77	56	82	49	114			
4	Pyridoxine	38	63	85	76	41	21	121	59
5	Pyridoxine	20	52	65	55	77	33	113	105
6	Pyridoxine, tryptophan	38	58	83	75	32	21	133	77
7	Pyridoxine, tryptophan	38	60	82	72	45	21	120	67
8	Ethyl linoleate	38	58	77	70	37	21	103	47
9	Linoleate, pyridoxine	38	55	71	62	56	21	124	100

All the other rats, after a period of depletion, were allowed to recover under various supplements. Nos. 4 and 5 received pyridoxine; Nos. 6 and 7, pyridoxine and tryptophan; No. 8, ethyl linoleate; and No. 9, linoleate and pyridoxine. All except No. 5 were 38 days on the deficiency diet, the same period as was rat No. 2, which was therefore considered the control. They were fed the supplements for 21 days. No. 5 was apparently more sensitive to the deficiency. In 20 days on the diet its weight loss was so great and its symptoms so severe that it was transferred to the supplemented diet on which it was kept for 33 days. It was, therefore, about the same age as the rest when killed.

## RESULTS

In the early period of the deficiency all rats underwent an initial gain in weight, a plateau and a loss. Table I gives the weights of the rats at weaning when they were put on the deficiency diet B (column *b*), the maximum (column *c*) and final weights (column *d*) during the period of deprivation, together with the weight lost expressed as percentage of initial gain (column *e*). Rat No. 1, which was on the deficiency diet for only 20 days, lost all of its initial gain. No. 2, although on the diet for 38 days and with severe symptoms, lost only 42% of its original increase. No. 3, on this diet 77 days and in the terminal stage of the deficiency, lost 114% of its increase. The others, except No. 5, gained from 16 to 25 g., with subsequent loss of 32–56% of their initial gains. No. 5 increased only 13 g. and lost 10.

Upon supplementation, alleviation of symptoms of all the rats was striking. Rat No. 4 was cured except for dry hind paws, a slight thinness of the hair and priapism. Rat No. 5 retained only a barely detectable dryness of the hind paws. Nos. 6, 7 and 8 displayed this same slight dryness of the hind paws and priapism. Six and 7 retained also some lachrymation. Except for priapism, No. 9 was completely cured in the 3 weeks of supplementation.

Table I also gives the final weights attained during the period of supplementation (column *g*) together with gain expressed as percentage of weight at the beginning of the supplemented diet (column *d*). The rats gained from 33 to 62 g.—No. 9, receiving both supplements, attaining the greatest increase, 62 g., and No. 8, receiving linoleate alone, undergoing the least, 33 g.

In Table II are given the analyses of the lipids of the rats of Table I. It may be seen that there was a small amount of total fat in the rats even in the deficiency stages (rats 1, 2, 3), with a slight decrease of questionable significance in amount as the condition advanced, 1.5, 1.1 and 1.0 g. On the supplements, all the rats gained in total fats in amounts ranging from 6 to 7 g. Their fat now constituted from 6 to 10% of the carcass weight as contrasted with 2–3% in the deficient animals. Hence, fat was being regenerated more rapidly than the other major body constituents.

The increase in total fats was spread over all fractions, the greatest gain being in the monounsaturated fatty acid group, although the increase expressed as percentage of the original content was somewhat



greater in the saturated fatty acid series. The di-, tri- and tetraenoic acids all showed increases above the very low values characteristic of the rats in advanced deficiency. The gain was most marked in the trienoic group.

TABLE II

*Analyses of Rats (1, 2, 3) Depleted of Fats for 20, 38 and 77 Days. Nos. 4 and 6-8 Were Depleted for 38 Days, No. 5, 20 Days. Supplements*

*Fed for 21 Days (No. 5, 33 Days)*

Nos. 4 and 5 received pyridoxine; Nos. 6 and 7, pyridoxine and tryptophan; No. 8, ethyl linoleate; No. 9, ethyl linoleate and pyridoxine. No. 2 is control.

Rat No.	Carcass wt.	Total lipids	I.V.	Non-saponifiable lipids	Saponifiable fatty acids	Saturated fatty acids	Monoenoic fatty acids	Di-, Tri-, Tetraenoic fatty acids			
								Total	Di-enoic	Tri-enoic	Tetra-enoic
	g.	g.		mg.	g.	g.	g.	mg.	mg.	mg.	mg.
1	47.0	1.48	81.5	298	1.18	0.41	0.65	118	30	21	68
2	59.0	1.09	75.2	87	1.00	0.33	0.61	64	16	4	44
3	45.6	1.02	94.5	49	0.97	0.09	0.83	55	16	7	33
4	111.0	7.06	61.9	338	6.67	2.61	3.83	220	33	93	93
5	109.1	7.77	65.0	265	7.51	2.64	4.62	259	78	71	110
6	119.7	8.16	66.7	321	7.84	2.61	4.99	239	61	99	76
7	107.6	8.05	66.3	410	7.64	2.51	4.89	249	75	75	98
8	95.2	7.41	67.0	223	7.18	2.41	4.46	309	172	75	108
9	120.5	11.47	57.8	256	11.22	4.52	6.40	301	157	29	135
Av. percent-age increase		8.32 669		321 269	8.01 701	2.88 773	5.03 725	263 311			

There was also a marked increase of the non-saponifiable fraction (column 5).

## DISCUSSION

At this stage of the double deficiency disease, caused by the withdrawal of both essential fat and pyridoxine from the diet, the symptoms can be materially alleviated by either ethyl linoleate or pyridoxine alone, as reported by earlier investigators. In all cases there was a clearing of the paw symptoms and the acrodynia of the snout and chin. Only slight scaliness of the hind paws remained. Attention should be

called to the short period of supplementation, 21 days, as possible explanation of the incomplete cure, since Burr (19) states that 4-6 weeks are required for complete disappearance of all symptoms under linoleate feeding. Rat No. 9, receiving both essentials, had normal feet.

The additional symptom of priapism persisted in all the rats but one, No. 5. Although this abnormality has not been mentioned in connection with either the syndrome of essential fatty acid deficiency or that of the florid acrodynia of pyridoxine, it is known to occur late in other deficiencies.

Growth, as reflected by weight gained, accompanied the disappearance of the symptoms. All the supplemented animals, except Nos. 8 and 9, gained 45-58 g. The greater increase of No. 9 receiving both supplements, and the lesser gain of No. 8 on the linoleate-supplemented diet, suggests that pyridoxine plays a more decisive role than linoleic acid in furthering growth.

The disease is characterized by low stores of fat, very low non-saponifiable lipids and small amounts of the highly unsaturated fatty acids. The unsaturation of the total fats increased as shown by the high iodine values of the depleted fats. This finding is in line with those of Longenecker, Gavin and McHenry (20) and Quackenbush and Steenbock (8). The first investigators, above, obtained elevated iodine values in the lipids of rats depleted by fat- and total vitamin B-free diets. Quackenbush and Steenbock, omitting pyridoxine and pantothenic acid, gave thiamine and riboflavin to rats on a fat-free diet and obtained high iodine values. Since the iodine value of the body fats of depleted rats, 1, 2, and 3, in Table II, were obtained on a complete diet except for pyridoxine and fat, the conditions for producing the higher unsaturation are here more specifically defined.

Between the advanced stage of the disease and the terminal condition the fats of the animals underwent practically no change in amount or analysis. These small values may be the lowest compatible with life.

The total fats increased with the recovery of the animals and were approximately the same whether the supplement was pyridoxine, pyridoxine and tryptophan, or ethyl linoleate. When linoleate and pyridoxine were administered together, the increase in fat was greater. Iodine values fell approximately the same amount in all animals except in the rat receiving both supplements, No. 9, which had the lowest value. This fall in iodine value indicates that fats other than the

highly unsaturated ones participate in the immediate response to the introduction of the supplements.

Recovery from the deficiency led to increases in all fractions of the fat, regardless of which of the two supplements was fed. This increase extended to the higher unsaturated fatty acids and was distributed fairly evenly over its three fractions. Rats 8 and 9, which received ethyl linoleate had their greatest increase in the dienoic acid fraction. This difference amounts to the equivalent of three days' intake, 30 mg./day.

As pointed out above, a certain reserve must be maintained in the interpretation of results based on spectroscopic analyses, since the characteristic absorption at each band is not specific for one degree of unsaturation, but a considerable overlapping occurs. The absorption at 2340A includes that due not only to linoleic acid, but also to any other dienoic acid, if present, together with low degrees of absorption from more highly unsaturated acids. The absorption at 2340A due to the tri- and tetraenoic acids has been corrected for in these determinations of dienoic acids but not that due to other polyenoic acids. Since these more highly unsaturated polyenoic acids are present, even under long deprivation of fats (Burr, private communication), and since the amount of their absorption at 2340A is unknown, too strict an interpretation of the absolute values of our determination must be avoided. According to Hilditch (21, p. 73), these highly unsaturated fatty acids have their origin in dietary fats; according to Nunn and Smedley-MacLean (22) they arise secondarily from linoleic and linolenic acids. None of these have been administered to those rats receiving pyridoxine as the only supplement, and hence it is improbable that they could account for the marked increase in the absorption in the region characteristic of dienoic acids.

It is difficult to reconcile these increases in dienoic acids with the results of Bernhard *et al.* (23) who, by the use of heavy water in the tissue fluids of rats, found no synthesis of linoleic or linolenic acids. While no explanation of the discrepancy is attempted at present, some differences between their attack of the problem and that in this study may be pointed out.

Bernhard's experiments were carried out with adult rats averaging 250 g. in weight, while these were done with young rats in the rapidly growing stage. These animals were weaned at 30 days, were on the deficiency regime for 38 days and on the supplemented diet for 21

days. They were, therefore, 83 days of age when sacrificed. During the 21 days of the supplemented diet they gained, on the average, 34 g. Bernhard's lost, on the average, 5.2 g. Of the 10 rats used by him, one gained 14 g., one 15 g. and one 5 g. All the rest lost from 0 to 57 g.

The average duration of his experiments was shorter, 9 days, with a range of 3–21 days. The three rats which gained in weight were on the experiments 6, 6 and 9 days.

His experiment may also be contrasted with this in that no preliminary deficiency was created, while in this study the dienoic content of the body fats was reduced to approximately 4 mg.

These factors would not have prevented the appearance of deuterium in the unsaturated fatty acids if there exists a rapid equilibrium between the stored acids and units from which they are synthesized. But other evidence points to slow metabolic processes involving these fat constituents. Burr and Burr (1) found that 70–90 days were required to produce skin changes in young rats on a fat-free diet containing all the B-vitamins. In adult rats typical skin symptoms appear only after an even more lengthy depletion period. The evidence from previous investigations, therefore, suggests that this system, although essential to growth, is metabolically sluggish compared with the more highly reactive saturated fatty acids.

Table III gives the fat content of the rats after supplementation, the net gain in fat and in body weight. In the final column are given the

TABLE III  
*Comparison of Fat to Total Body Synthesis in Rats during  
Pyridoxine and Linoleate Supplementation*

Rat No.	Supplement	Total fat	Fat incr.	Wt. incr.	Fat incr. Per cent wt.
		g.	g.	g.	
2		1.1			
4	Pyridoxine	7.1	6.0	45	13.3
5	Pyridoxine	7.8	6.7	58	11.5
6	Pyridoxine + tryptophan	8.2	7.1	58	12.2
7	Pyridoxine + tryptophan	8.1	7.0	48	14.6
8	Linoleate	7.4	6.3	33	19.1
9	Linoleate + pyridoxine	11.2	10.1	62	16.3

fat increases expressed as percentages of the total gains. It may be seen that Nos. 8 and 9, receiving linoleate, have a higher percentage of fat synthesis than those receiving only pyridoxine, while 8, receiving linoleate, has an even greater percentage of fat alone than 9. Although the linoleate-fed animal (8) synthesized approximately the same amount of fat it was unable to synthesize its other body constituents at the same rate as those receiving pyridoxine (4-7). These findings indicate that pyridoxine is more powerful in furthering growth, while linoleic acid is more concerned in the synthesis of fat.

### SUMMARY

1. Animals in deficiency states caused by exclusion from the diet of fat and pyridoxine were studied for their response to ethyl linoleate, to pyridoxine, to pyridoxine and tryptophan, and to ethyl linoleate and pyridoxine.

2. Relief from the deficiency was brought about by either linoleate or pyridoxine, and was characterized by alleviation of symptoms, resumption of growth, and increase in body fat.

3. Growth was greater on pyridoxine alone than on linoleate alone. When both essentials were administered together, growth was further increased.

4. The fat produced in animals relieved by pyridoxine was of approximately the same amount as in animals relieved by ethyl linoleate. In the latter instance, the percentage of fat formed was greater. Where both supplements were used together, the fat increase was significantly greater than where either was used separately.

5. Tryptophan did not have any influence.

6. All fractions of the fat of these animals were increased, including the highly unsaturated fatty acids. On the pyridoxine supplement the increase was distributed almost equally among the di-, tri-, and tetra-enoic fractions. When linoleate was fed, the dienoic fraction was more elevated.

7. Although linoleic acid appears to share in the general elevation following pyridoxine feeding, the extent of its participation cannot be accurately evaluated until more specific methods of estimation and identification can be devised.

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## A *Lactobacillus* of Cecal Origin Requiring Oleic Acid

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Received January 20, 1947; Revised July 7, 1947

### INTRODUCTION

The stimulatory effect of oleic acid on the growth of *Lactobacilli* in the presence of suboptimal amounts of riboflavin has been demonstrated by Strong and Carpenter (1). Bauernfeind *et al.* (2) showed the same effect on pantothenic acid assays. Williams and Fieger (3) reported that oleic acid had a marked stimulatory effect in biotin assays and later found (4) that *L. casei* could be grown in an essentially biotin-free medium, provided oleic acid was present. Guirard *et al.* (5) found several fatty acids, including oleic acid, were capable of replacing the nutritional function of acetate with several *Lactobacilli*. *Corynebacterium diphtheriae* and *Clostridium tetani* have been demonstrated to require oleic acid by Cohen and Mueller (6) and Feeney *et al.* (7), respectively.

In the course of studying the nutrition of cecal *Lactobacilli*, isolated from rats fed on a highly purified diet, a strain was found which was unable to grow on a synthetic medium considered complete for *L. casei* (Table I).<sup>1</sup> None of the known bacterial stimulants available in the laboratory furnished the essential factor.

The method of approach in determining the nature of the missing factor was to find the natural supplements which would permit growth when added to the synthetic media. One of these was fractionated to determine the chemical properties of the essential factor. Graded dilutions of the supplement were used in constructing the standard curve for assaying the potency of the fractions made from it.

<sup>1</sup> The amount of pyridoxine is suboptimal if used without subsequent heating in the presence of amino acids (Snell and Rannefeld, 11). In this case, enough "pseudo-pyridoxine" was produced during autoclaving so that high levels of pyridoxine gave no increased response.



TABLE I

*Double Strength Basal Medium*

Hydrolyzed casein.....	2.5	gm.
L-Tryptophan.....	50.	mg.
L-Cystine hydrochloride.....	50.	mg.
Adenine sulphate.....	5.	mg.
Guanine hydrochloride.....	5.	mg.
Uracil.....	5.	mg.
Thiamine chloride.....	100	γ
Pyridoxine.....	100	γ
d-Ca pantothenate.....	100	γ
Riboflavin.....	200	γ
Nicotinic acid.....	50	γ
p-Aminobenzoic acid.....	40	γ
Biotin.....	0.75	γ
Pteroylglutamic acid.....	10	γ
Inositol.....	50	γ
Glucose.....	5	gm.
Sodium acetate.....	3	gm.
Speakman Salts A <sup>1</sup> .....	2.5	ml.
Speakman Salts B <sup>2</sup> .....	2.5	ml.
Distilled water to.....	250	ml.
pH.....	6.6-6.8	

<sup>1</sup> Salts A contained: 25 g. of  $K_2HPO_4$  and  $KH_2PO_4$ /250 ml. of solution.

<sup>2</sup> Salts B contained: 10 g.  $MgSO_4 \cdot 7H_2O$  and 0.5 g. each of  $FeSO_4 \cdot 7H_2O$ ,  $MnSO_4 \cdot 4H_2O$  and  $NaCl$ /250 ml. of solution.

## EXPERIMENTAL

*Organism Used*

The organism is a gram positive rod, occurring in short chains or singularly, never motile. It is the dominant flora in the cecum of rats fed on a highly purified diet. On Bacto tomato juice agar, colonies are of the X type, *e.g.*, having delicate filament outgrowths, giving a rough wooly appearance. It grows well at 45°C. but not at 50°C. At 30°C. it will not curdle milk in less than 7 days. On lactose-yeast-peptone agar, it tolerates a pH of 7.8, a sodium chloride concentration of 2.5%, and a phenol dilution of 1:300. In litmus milk, the curd is firm and the litmus is slowly reduced. Glucose, sucrose, lactose, and salicin, but not mannitol, are fermented. According to the criteria presented by Curran *et al.* (8) and Sherman and Hodge (9), the organism has the characteristics of *L. acidophilus*.

*Basal Media*

The basal medium used in all experiments except where specifically stated is listed in Table I.

The purines and pyrimidines were combined in one solution and stored in the refrigerator. The vitamin supplements were stored in brown bottles under toluene with exception of biotin, which was preserved in 25% alcohol.

### *Testing Procedure*

Neutralized supplements were added to 5 ml. of the double strength basal medium and the final volume brought to 10 ml. The testing procedure was essentially that of Snell and Wright (10). The standard curve was constructed by plotting the titrations against graded concentrations of the crude material from which the fractions were made.

### *Measurement of Growth Response*

After 72 hours incubation at 37°C., culture tubes were titrated with 0.1 N NaOH using bromthymol blue as the indicator. Potency of the source material was determined by the amount of acid produced. Recovery of activity in the chemical fractions were calculated from the standard curve of the crude substance.

## RESULTS

### *Response to Vitamins and Bacterial Growth Stimulants*

No growth occurred when the following were added to the basal medium: thymine, orotic acid, choline, asparagine and glutamine. The following vitamins: thiamine, pyridoxine, calcium pantothenate, riboflavin, nicotinic acid, *p*-aminobenzoic acid, biotin, and pteroylglutamic acid, gave no response when added to the basal medium at five times the level stated in Table I.

### *Response to Natural Supplements*

Soybean protein, crude liver extract, Bacto peptone, yeast extract (Difco), Vitab, Indian molasses, concentrated orange juice, apple

TABLE II

#### *Response to 500 $\gamma$ of Active Supplements*

Material tested	Titration of 72-hour culture (ml. 0.1 N NaOH)	Titration of uninoculated sample. (ml. 0.1 N NaOH)
Pressed liver cake	5.6	0.8
Bacto heart infusion	4.2	0.9
Asparagus juice	2.1	1.1
Fish solubles	3.6	0.8
Distillers solubles	3.6	0.8

honey, pentanucleotides, corn steep liquor, papain digested liver cake, and purified anti-pernicious anemia extract<sup>2</sup> showed no response at levels of 500  $\gamma$ /ml.

Table II gives a list of active natural supplements and their activity. Fish solubles was chosen for further study because of its solubility.

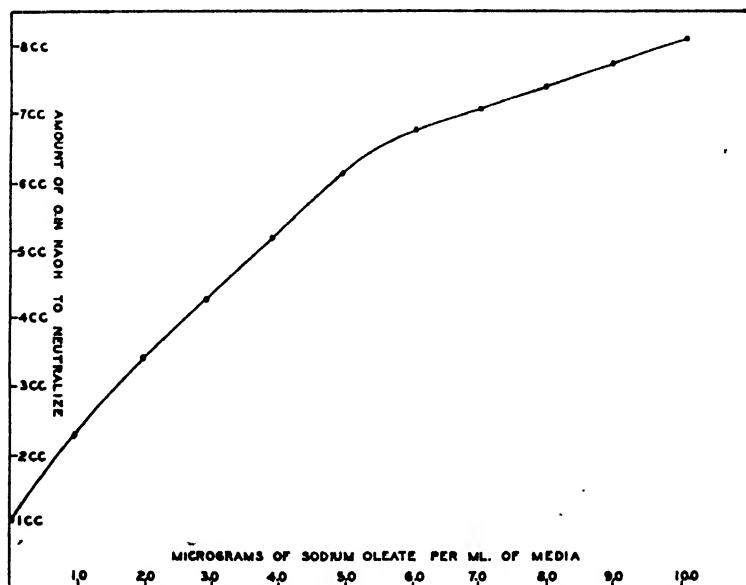


FIG. 1. Response to fish solubles.

#### *Experiments Indicating the Nature of the Active Substance*

Ten g. samples of fish solubles were diluted with 20 ml. of water and adjusted to pH 2, 7 and 9. They were then autoclaved at 15 pounds pressure for 2 hours. The samples which were autoclaved at pH 2 and 7 showed no change in activity. However, the sample autoclaved at pH 9 had double the activity of an untreated sample. When the alkali-treated sample was adjusted to pH 2, the active material was readily extracted by petroleum ether. The same results were demonstrated with distiller's solubles.

In addition, a solution of fish solubles was adjusted to pH 4.5 and

<sup>2</sup> Lederle Laboratories 15 unit liver.

filtered. The activity remained in the fatty acid residue on the filter paper.

Data from these experiments are given in Table III.

TABLE III  
*Response to Fractions of Fish Solubles and Distillers Solubles*

Fractions tested (number of times assayed given in parenthesis)	$\gamma$ Equivalents of fish solubles	Activity (fish soluble equivalents)	
		Range	Average
1. Alkali-treated fish solubles (5)	100-400	1.8 -2.2	1.90
2. Acid pet. ether extract of 1 (5)	200-600	1.1 -1.6	1.44
3. Acid pet. ether residue of 1 (5)	800-1600	.11- .43	0.33
4. pH 4.5 ppt. of fish solubles (3)	100-600	1.06-1.30	1.12
5. pH 4.5 filtrate of fish soluble (2)	600-5000	inactive	0
6. Alkali-treated distillers solubles (2)	100-500	1.70-2.20	1.95
7. Acid pet. ether extract of 6 (2)	100-400	1.60-2.00	1.80
8. Acid pet. ether residue of 6 (2)	800-4000	0 - .31	0.15

#### *Response to Sodium Oleate*

The above data indicated the growth factor might be a fatty acid (1). Na oleate was assayed and found to be 275 times as active as fish solubles. Fig. 2 shows the response of the organism to sodium oleate.

#### *Response to Purified Sodium Oleate*

To eliminate the possibility that the response was due to some water-soluble impurity in the sodium oleate, the following process was applied. An aqueous solution of the salt was adjusted to pH 2 and extracted with petroleum ether. The oleic acid was re-extracted from the petroleum ether solution with 0.01 N NaOH. Full activity was obtained in the purified substance.

#### *Response to Acetate*

The sodium acetate in the basal medium was varied from 0.6 to 2.0% with no effect on the response to sodium oleate. Complete substitution of the acetate with a phosphate buffer did not affect the sodium oleate assay.<sup>3</sup>

<sup>3</sup> Growth determined turbidimetrically.

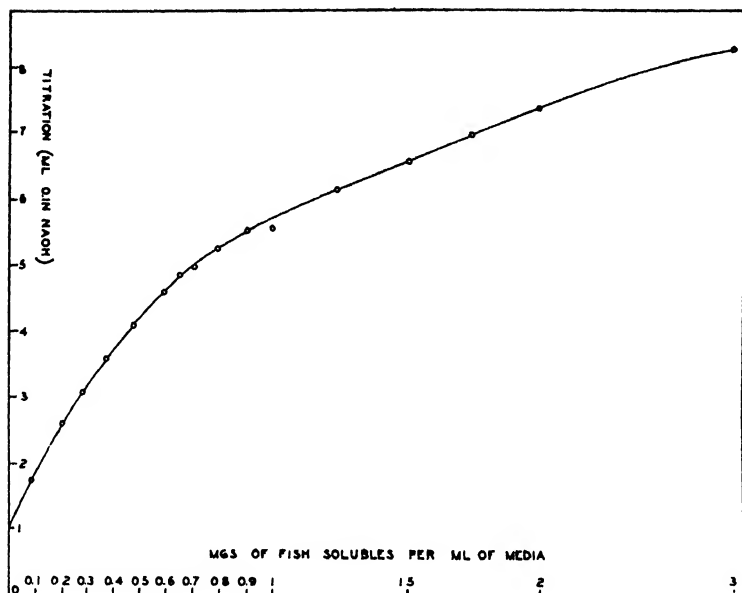


FIG. 2. Response to untreated sodium oleate.

#### *Response to Other Oleic Acid Derivatives*

Lecithin was found to have 4% the activity of sodium oleate. Sorbitol monooleate (Tween 80) at 50  $\gamma$ /ml. level was 10% as active as sodium oleate; at higher levels it proved toxic to the organism. Sorbitol monostearate (Tween 60) was inactive.

#### *Maintenance of the Organism on Basal Medium Containing Sodium Oleate*

The *Lactobacillus* described in this paper was passed through 30 daily transfers of the basal synthetic media containing 5  $\gamma$  of sodium oleate/ml. with no decrease in response. Subcultures into basal medium without sodium oleate never have shown growth.

#### DISCUSSION

This investigation has shown that oleic acid is an essential factor in the nutrition of the *Lactobacillus* described above. Continuous subculture on a synthetic medium is possible only in the presence of the substance.

The properties of the factor as present in fish solubles are those of a fatty acid. Oleic acid gives maximum growth of the organism when used to replace fish solubles.

Other investigators have demonstrated oleic acid to be a growth stimulant of *Lactobacilli* with suboptimal amounts of riboflavin, pantothenic acid and biotin, but maximum growth could be obtained in its absence. The strain of *Lactobacillus* used in this study, does not grow in the absence of sodium oleate when all of the vitamins are added in a 5-fold excess of what is ordinarily considered sufficient for *Lactobacilli*. Several rich vitamin sources, such as crude liver extracts, yeast and Vitab, fail to induce growth. The sodium oleate was purified to insure that the growth stimulation was not due to a water-soluble impurity.

Guirard *et al.* (5) found evidence that certain *Lactobacilli* may use acetate to synthesize essential lipoidal material, including fatty acids. The *Lactobacillus* used in this investigation did not require acetate, but could not grow in the absence of oleate.

#### SUMMARY

1. An essential growth factor, present in fish solubles, necessary for the growth of a *Lactobacillus* isolated from the cecum of a rat, was demonstrated to be oleic acid.

2. The *Lactobacillus* could be transferred repeatedly on synthetic media only in the presence of sodium oleate.

3. Its requirement for oleic acid could not be modified by high levels of sodium acetate, biotin, riboflavin, pantothenic acid, or any of the other known factors tried.

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# Polarographic Studies of Proteins and Their Degradation Products. II. Normal Values of the "Protein Index"<sup>1</sup>

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Received May 1, 1947

## INTRODUCTION

In a preceding paper (1) we proposed the *protein index* as a convenient method of characterizing and comparing results obtained during the cobalt-catalyzed polarographic reduction of cystine- or cysteine-containing proteins and polypeptides. The protein index is a function of the ratio of the polarographic wave-heights obtained with two different tests, the *filtrate* and *digest* tests, which are carried out under identical experimental conditions on the same blood sample. Because these two tests show deviations with abnormal bloods which are in opposite directions, the protein index magnifies these deviations from normal and makes the polarographic analysis more sensitive. The protein index is especially well suited for routine analyses of blood proteins because it is practically independent of small variations in room temperature and is little influenced by considerable changes in the drop time and the drop size of the dropping mercury electrode (1).

The purpose of the present paper is to demonstrate that the protein indexes of normal individuals are remarkably constant over long periods of time and fall within a narrow range of values. This normal range, therefore, can serve as a basis for the comparison of abnormal bloods which need not be analyzed simultaneously with a "normal" as has

<sup>1</sup> The authors gratefully acknowledge their indebtedness to Mrs. Sarah D. Gardiner for financial support of this investigation.

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heretofore been necessary. In a subsequent paper it will be shown that bloods from patients with a variety of diseases differ sufficiently from the normal range to make the protein index a useful indicator of abnormalities.

## METHOD

### *Recommended Procedure*

About 5 ml. of fresh venous blood is placed in a centrifuge tube containing a few crystals of potassium oxalate and centrifuged for 15 minutes. With an Ostwald-Folin or an Ostwald-Van Slyke pipette four 0.50 ml. samples of the supernatant plasma are taken off and placed in 4 test tubes. This provides sufficient samples for duplicates of the following filtrate and digest tests.

*Filtrate Test.* To each of two 0.50 ml. samples of oxalated plasma add 1.00 ml. of water and 0.10 ml. of 1.0 *N* potassium hydroxide.<sup>3</sup> Mix well and let stand for 30 minutes at room temperature, then add 1.00 ml. of 20% sulfosalicylic acid and again mix thoroughly. Exactly 10 minutes after the addition of the protein precipitant, pour the suspension through a Whatman No. 5 filter paper (5.5 cm. diam.). The resulting filtrate should be clear; it is fairly stable and can, therefore, be analyzed when convenient. For such analysis 0.50 ml. of the filtrate is added to 5.0 cc. of a buffered solution containing trivalent cobalt (see below) and polarographed immediately in a 10 ml. beaker, open to air, starting at  $-0.8$  v. (vs. S.C.E.).

*Digest Test.* To each of two 0.50 ml. samples of oxalated plasma add 0.50 ml. of water and 0.25 ml. of 1.0 *N* potassium hydroxide.<sup>3</sup> Mix thoroughly and let stand for 30 minutes at room temperature. Of the resulting alkaline digest, 0.05 ml. is added<sup>3</sup> to 10.0 ml. of a buffered solution containing divalent cobalt (see below) and polarographed immediately in a 20 ml. beaker, open to air, starting at  $-0.8$  v. (vs. S.C.E.).

Since the analyses described depend to a large extent on careful timing of the digestion and protein precipitation, it is necessary to plan series analyses very carefully. With the polarograph which we used, each curve could be drawn in 3 minutes. We therefore made our potassium hydroxide additions at intervals of 4 minutes and could thus run 12 digest tests in 1.5 hours. This includes the 30 minute period of digestion for the first sample, which time can be used to advantage in getting the equipment and solutions ready. We usually prepared the filtrates a little ahead of the digests, but carried out their polarographic analysis immediately after that of the digests. In this way one is assured of the primary requirements for the protein index, namely, identical experimental conditions for the two tests. With good planning, it is not difficult to determine the protein index of 12 bloods (or of 6 bloods in duplicate) in 3.5–4 hours, starting with whole blood.

For a description of the polarographic technique and terminology, the reader is referred to the literature (2–5). Typical polarographic

<sup>3</sup> To pipette these quantities accurately, we found it advantageous to use Kahn pipettes C and D.

curves obtained with these two tests are illustrated in Fig. 1. They all show a cobalt wave (with or without a maximum, depending on the concentration of the polypeptides which act as suppressors) followed by the double-wave which is characteristic of sulfhydryl-containing proteins and polypeptides. In evaluating the curves we have measured (as indicated in Fig. 1) the height of the double-wave as the difference

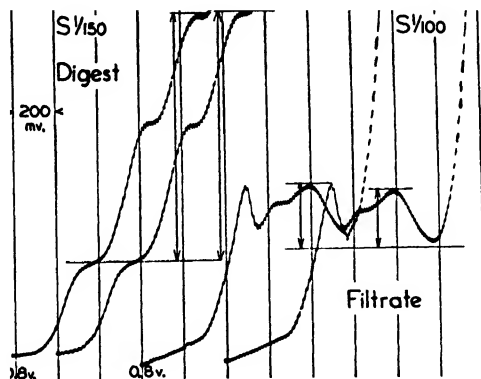


FIG. 1. Polarogram illustrating curves obtained with the digest and filtrate tests, in duplicate.

between the peak of the second part of the double-wave and the level of the cobalt wave. In the case of the digest test this can be done readily because the cobalt wave has no maximum. In the case of the filtrate test, however, the height of the cobalt wave has to be determined by a blank experiment. Of course, this height remains unchanged under standard conditions so that only occasionally a blank test needs to be made for checking purposes. In Fig. 1 we have drawn in the known cobalt wave height for the filtrate test.

From such curves, the protein index is determined as follows. The wave height obtained in the filtrate test is divided by that found in the digest test for the same sample of plasma under identical experimental conditions. This ratio, multiplied by a factor, which depends on the galvanometer sensitivities used for the two tests, then gives us the protein index. The factor is 15 if the wave heights are expressed in microamperes, or if they are expressed in mm. and the galvanometer sensitivities used in the two tests are the same. However, if the filtrate wave is recorded at a higher galvanometer sensitivity than the digest

wave, as is more often the case, the factor will include a correction for this difference in sensitivity and be smaller (*e.g.*, if, as in Fig. 1, the digest wave is taken at 1/150 and the filtrate wave at 1/100 of maximum galvanometer sensitivity, the factor is 10).

The following solutions are necessary to carry out these tests.

*Stock Solutions.* Only the best commercially available grade of reagents should be used.

- (a) 1 *N* KOH: To 66 g. KOH (Reagent grade, minimum KOH = 85%) add enough water to make 1 l. of solution.
- (b) 20% Sulfosalicylic acid: 20 g. sulfosalicylic acid is dissolved in water and diluted to 100 cc.
- (c) 0.008 *M* Cobaltous chloride: Dissolve 1.9036 g.  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  in water and dilute to 1 l.
- (d) 0.01 *M* Hexamminocobaltic chloride: Dissolve 1.337 g.  $\text{Co}(\text{NH}_3)_6\text{Cl}_2$  in water and dilute to 500 cc. Contrary to the observations of Crossley *et al.* (6) we have found that this solution is quite stable for our purposes. A solution 3 years old, which had become slightly alkaline and from which had settled out a film of brownish material, gave cobalt and protein waves which were but 2% smaller than those obtained under identical conditions with a fresh solution.
- (e) 1.0 *N* Ammonium chloride: To 53.5 g.  $\text{NH}_4\text{Cl}$  add enough water to make 1 l. of solution.
- (f) 1.0 *N* Ammonium hydroxide: If the commercially available ammonia water is quite fresh, about 34 cc. of it, diluted with water to 500 ml. will give an approximately 1 *N* solution. Since most stocks which have been on the shelves for some time have lost ammonia, it is best to prepare a slightly more concentrated solution at first and adjust it, to 1.0 *N* by dilution after its titer against a standard acid has been determined. Frequent checks should be made to be sure of the constancy of this solution which is best kept in an ice box.

*Test Solutions.* These solutions contain volatile ammonia and should be prepared and kept in glass-stoppered bottles. They should not be exposed to the atmosphere any longer than absolutely necessary.

*Trivalent Cobalt Buffer* (for the filtrate test): 0.001 *M* hexamminocobaltic chloride, 0.1 *N* ammonium chloride and 0.8 *N* ammonium hydroxide. It is prepared from 10.0 ml. of solution (d) to which are added 10.0 ml. of solution (e) and 80.0 ml. of solution (f). With proper precaution against loss of ammonia (storage in an ice box and infrequent exposure to the atmosphere), this solution may be kept for several months. On the other hand, the loss of ammonia from an open beaker is so rapid that the 5 ml. needed for the polarographic analysis should be pipetted immediately before the filtrate is added.

*Divalent Cobalt Buffer* (for the digest test):  $1.6 \times 10^{-3}$  *M* cobaltous chloride, 0.1 *N* ammonium chloride, and 0.1 *N* ammonium hydroxide. It must be freshly prepared for each series of analyses by taking 20.0 ml. of solution (c), and adding 10.0 ml. of solution (e), 60.0 ml. of distilled water, and 10.0 ml. of solution (f). Care must be taken to add the ammonium hydroxide last, to prevent precipitation of the cobalt.

### *Polarographic Equipment*

The polarograms were automatically recorded with a Heyrovsky-Shikata Polarograph, Model VIII.<sup>4</sup> The galvanometer had a maximal sensitivity of  $2.1 \times 10^{-9}$  A./mm./m., which was suitably reduced by means of a special shunt. The experiments were carried out at room temperature. The anode was a saturated calomel half-cell, connected to the test solution by means of a saturated potassium chloride and potassium nitrate bridge (4).

The anode, bridges and dropping mercury electrode were clamped firmly in position on a special stand. One bridge was attached to the dropping mercury electrode by means of a 2-hole rubber stopper in such a way that one end of it was level with the tip of the dropping mercury electrode. The test solutions in small beakers could then be analyzed in rapid succession by moving each beaker onto a little platform beneath the capillary. Between different samples, the capillary and bridge were washed with distilled water and dried with filter paper, thus preventing any measurable contamination of the solutions. Removal of dissolved oxygen by passage of an inert gas through the solutions is not necessary and would actually be disadvantageous because it would simultaneously wash out some of the ammonia.

Most of the results presented here were obtained with a dropping mercury electrode with a capillary which had been cut off improperly. When the capillary was mounted vertically, the cut surface formed an angle of about  $20^\circ$  with the horizontal. Nevertheless, because of the rigidly fixed position of the capillary, the polarographic curves were reproducible, and this fault was not discovered until a special study of the characterization of capillaries was undertaken (7). This capillary had the advantage that smaller drops were formed than one would obtain with a normal capillary of the same characteristics, thus reducing the recorded galvanometer oscillations on the polarogram and producing more points on the drawn curves. However, because a slight change in the position of this type of capillary could produce variations in the results, we do not recommend it and have switched to correctly cut capillaries, the normal behavior of which was ascertained by inspection and by a method described elsewhere (7). In agreement with our earlier observations (1), the protein index of a given plasma was essentially the same for different capillaries.

### DISCUSSION OF THE METHOD

The filtrate and digest tests which have been combined in the determination of the protein index are modifications of some of the numerous procedures which have been recommended from time to time by Brdička (8-10) for the diagnosis of cancer. This combination of two tests not only makes the method relatively independent of

<sup>4</sup> Inasmuch as the measured wave-heights are often just peaks on the polarographic curves (see the filtrate curves of Fig. 1), an automatic recording device is of considerable advantage. However, if measurements are made with a manually operated apparatus at sufficiently small voltage increments, results of the same accuracy can be obtained with it (see Müller (4), p. 106).

experimental conditions, but also increases the sensitivity of the method, because a deviation from normal produces an increase in the filtrate and a decrease in the digest wave height (1).

In agreement with Brdička (9) we found 30 minutes to be the best and least critical time for the alkaline denaturation. The polarographically active material increases rapidly during the first 20 minutes, more slowly to its maximum value around 30 minutes, and decreases thereafter gradually with longer periods of digestion.

We have also investigated a test proposed by Brdička (8, 9) in which potassium iodoacetate is added to combine with the sulfhydryl group and leave only disulfidic groups to react at the dropping mercury electrode. We analyzed over 200 plasmas by our standard digest procedure and again by a digest test in which 0.5 ml. of 0.2 *M* potassium iodoacetate was added in place of the water. Fourteen of these plasmas we digested for a period of 45 minutes in the presence of iodoacetate (following Brdička's directions); the resulting values were  $83.4 \pm 1.8\%$  of those found in our regular digest test. To get a better basis for comparison we made the time of digestion of the remaining plasmas identical in the two tests, that is, equal to 30 minutes. The results of the iodoacetate tests were as follows: 52 plasmas from normal individuals  $93.7 \pm 0.5\%$  of normal; 155 plasmas from patients with a variety of diseases  $93.2 \pm 0.3\%$  and 8 plasmas from dogs  $88.4 \pm 0.7\%$  of the control values. We felt that there was not sufficient difference between normal individuals and patients to warrant the extra manipulation and, therefore, discontinued the iodoacetate additions.

In our standard filtrate and digest tests the alkaline denaturation of protein is carried out at different concentrations of the reactants. It seemed logical to attempt a simplification of the analysis by carrying out both tests at the same concentrations. A total of 89 normal and abnormal plasmas were consequently treated with 1.0 ml. of 20% sulfosalicylic acid after having undergone an alkaline denaturation for 30 minutes as in the digest test. Duplicate samples of these plasmas were also subjected to our usual filtrate test.

The results of these simultaneous analyses showed that practically no difference existed between the two procedures at low wave heights but that there was a definite tendency toward higher values with our usual filtrate test as the wave heights increased. For this reason, and because the alternate procedure did not produce as great a saving in time as expected, we have adhered to the filtrate test as described under "Method."

Practically all our analyses were carried out with oxalated plasmas, whereas Brdička's work was done with sera. Some preliminary observations showed that serum would give slightly higher waves than

plasma in both tests and that the protein index might become higher by as much as one unit. Citrated plasma gave results similar to those obtained with oxalated plasma. Defibrinated blood from which the corpuscles had been removed by centrifugation gave values close to those obtained with serum. The presence of erythrocytes, or marked hemolysis, suppresses to a large extent the polarographic waves in either test; the effect of slight hemolysis is negligible. The amount of oxalate which must be used is not critical; excess oxalate does not interfere with the analyses.

The protein index can be determined with as little as 0.4 ml. of plasma which can be obtained from 1 ml. of whole blood. We were thus able to study blood changes in rats, because that much blood can be repeatedly obtained by heart puncture. For the studies on man, which are described here, we preferred to take 5 ml. blood by venal puncture; the 2 ml. plasma obtained therefrom are sufficient to run both tests in duplicate with more conveniently measured quantities of reagents. The blood samples were collected with as little stasis as possible, without regard to time of day or food intake, since a preliminary study (made on a dog) showed only negligible variations in the index during the day.

It is best to work with fresh plasma, but blood or plasma that has been stored for a day or two in the refrigerator is still suitable, as long as there is no appreciable hemolysis. Further storage is detrimental; the index may be increased or decreased depending on the length of storage and possible contamination. Whenever it was impossible to analyze the bloods immediately upon receipt, we centrifuged the bloods and stored the plasma over night in a small test tube sealed with parafilm.

The reproducibility of the polarographic curves is perfect as long as the recording of the curves is repeated within 15 minutes. After that time the curves will become lower because of some loss of ammonia from the test solution. The accuracy of the results compares well with other polarographic procedures. It is not difficult to go through the whole procedure for duplicate plasma samples and obtain protein indexes which agree within 5%. Errors greater than that can usually be traced to faulty pipetting. The duplicate analyses shown in Fig. 1 may serve as an example. The two digest waves are 64.4 and 64.8 mm. high, while the two filtrate waves are 16.5 and 15.4 mm. high. The error in the latter is relatively large because the total wave height is small.

The protein indexes calculated for the highest and lowest values are 2.5 and 2.4 respectively. This relative insensitivity of the protein index to small errors in procedure and to small variations in the experimental conditions (1) makes the method suitable for clinical laboratories where specially trained help is not available.

## RESULTS

This report covers a total of 109 analyses made with blood from 32 volunteer medical students, nurses, staff members and visitors to the laboratory. They all considered themselves to be normal, had not had any recent illnesses and were "feeling fine." Of these analyses, 81, representing 18 individuals, were made with the abnormal capillary (No. 1) mentioned earlier, and the rest were made with a normal capillary (No. 11). The results are summarized in Table I.

The observed wave-heights have been evaluated and are given in microamperes. Comparison of the average currents observed in the two tests with the two capillaries shows that those obtained with capillary No. 11 are almost twice as large as those obtained with capillary No. 1. Nevertheless, the protein index calculated on the basis of these currents is independent of the capillary. When the means of the index values for the two capillaries were compared, it was found that the difference between them was 0.55 times the standard error of the mean and, therefore, not significant. Hence, this substantiates our claims for the protein index (1).

It may be seen from Table I that the protein indexes of different individuals may vary considerably but remain remarkably constant for any given individual. A maximum variation of 1.3 units is found in the index of Subject G. S., while the average variation in the index of any individual is 0.7 units. Values obtained over a period of more than a year on two individuals with relatively high indexes are given in Table II. The results shown for Subject O.M. are particularly interesting since the last two measurements were made after a period of over 4 years and with a different capillary; yet the index remained essentially constant. In view of this constancy, and to avoid a bias in our results in favor of those individuals whose plasma was analyzed most frequently, we have used the average values for each individual in comparing the protein indexes.

These averages bring out clearly a definite variability between

different "normal" individuals, which explains the quantitatively poor results of earlier tests in which the blood of *any* normal individual was

**TABLE I**

*Normal Filtrate, Digest and Protein Index Values of Men and Women*

Subject	Age at start	No. of blood samples	Period covered (months)	Current in $\mu$ A.				Protein index		Mean current in $\mu$ A./mm. <sup>2</sup>	
				Filtrate test		Digest test		Range	Mean	Filtrate test	Digest test
				Range	Mean	Range	Mean				
Abnormal Capillary No. 1											
Men:											
O. M.	32	10	22	3.97-5.27	4.65	17.2-19.6	18.5	3.3-4.3	3.8	4.35	17.3
R. F.	28	4	5	2.06-2.42	2.25	20.4-21.1	20.7	1.5-1.7	1.6	2.10	19.3
D. P.	25	4	4	2.92-3.36	3.07	17.7-18.1	17.9	2.4-2.8	2.6	2.87	16.7
J. D.	40	5	2	1.72-2.16	1.99	15.5-18.4	17.5	1.4-2.0	1.7	1.86	16.3
C. W.	36	2	1	1.72-2.26	1.99	18.5-19.6	19.0	1.3-1.8	1.6	1.86	17.8
E. D.	49	1			3.99		16.5		3.0	3.73	15.4
E. C.	42	1			2.79		17.3		2.4	2.61	16.2
J. S.	40	1			3.48		18.6		2.8	3.26	17.4
A. L.	42	1			2.23		18.4		1.8	2.08	17.2
Average					2.94		18.3		2.4	2.75	17.1
Women:											
M. D.	27	9	17	2.96-4.84	3.95	14.8-19.8	17.2	2.9-4.0	3.4	3.69	16.1
E. M.	26	3	10	2.50-2.94	2.79	16.8-18.5	17.6	2.2-2.5	2.4	2.61	16.4
E. V.	22	12	2	1.60-2.60	2.10	16.1-19.9	17.7	1.4-2.4	1.8	1.96	16.5
M. S.	22	11	2	1.16-2.84	1.62	16.4-19.3	17.9	1.0-2.3	1.4	1.51	16.7
G. S.	24	10	2	1.20-2.02	1.55	16.9-19.8	18.1	1.0-1.6	1.3	1.45	16.9
J. M.	21	3	1	1.41-1.64	1.51	14.3-17.1	15.6	1.3-1.7	1.4	1.41	14.6
I. G.	27	2	1	2.27-3.15	2.71	17.1-18.1	17.6	1.9-2.8	2.3	2.53	16.4
M. M.	33	1			3.78		16.6		3.4	3.54	15.5
E. S.	24	1			3.28		17.1		2.9	3.06	16.0
Average					2.59		17.3		2.3	2.42	16.1
Grand Average					2.76		17.8		2.3	2.58	16.6
Normal Capillary No. 11											
Men:											
O. M.	38	2	1	6.45-7.93	7.19	29.4-31.3	30.4	3.3-3.8	3.6	4.13	17.5
F. M.	22	4	1	4.63-5.51	5.14	27.6-29.0	28.2	2.5-3.0	2.7	2.95	16.2
B. S.	22	4	1	6.81-7.94	7.25	26.0-29.4	27.8	3.6-4.3	3.9	4.17	16.0
B. H.	20	4	1	4.86-6.08	5.33	26.3-29.6	27.9	2.8-3.1	2.9	3.07	16.0
J. S.	22	2	1	9.26-10.10	9.71	27.9-28.7	28.3	4.8-5.4	5.1	5.59	16.3
H. F.	21	4	1	4.01-5.84	4.98	28.2-29.9	29.0	2.1-2.9	2.6	2.86	16.7
R. J.	23	1			2.96		31.0		1.4	1.70	17.8
A. B.	41	1			3.94		31.0		1.9	2.27	17.8
B. W.	26	1			3.75		31.1		1.8	2.16	17.9
I. B.	23	1			5.29		29.0		2.7	3.04	16.7
A. M.	29	1			2.33		28.3		1.2	1.34	16.3
H. D.	27	1			3.72		28.1		2.0	2.14	16.1
Women:											
E. S.	22	1			2.24		30.6		1.1	1.29	17.6
M. F.	27	1			4.35		29.7		2.2	2.50	17.0
Grand Average					4.87		29.4		2.5	2.90	16.8



run as a control along with the unknown (9). Tropp (11), who recognized such variations in the normals and attributed them to differences in protein content, recommended that the latter be determined in each analysis. It is questionable, however, whether the refractometric

TABLE II  
*Variation in the Normal Filtrate, Digest and Protein Index Values  
of Two Individuals Over Periods of More Than a Year*

Subject O.M. (male)				Subject M.D. (female)			
Date	Current in $\mu\text{A./mm.}^2$		Protein index	Date	Current in $\mu\text{A./mm.}^2$		Protein index
	Filtrate	Digest			Filtrate	Digest	
Capillary No. 1				Capillary No. 1			
11/30/40	4.73	16.5	4.3	11/27/40	4.28	15.9	4.0
12/17/40	3.71	16.7	3.3	12/15/40	2.77	13.9	3.0
1/15/41	4.32	18.3	3.5	12/27/40	3.44	14.1	3.7
1/21/41	4.26	16.1	4.0	1/3/41	3.67	16.7	3.3
3/24/41	4.16	18.0	3.5	1/10/41	3.14	16.2	2.9
6/3/41	4.04	16.3	3.7	1/14/41	3.42	15.9	3.2
6/20/41	4.38	17.8	3.7	1/20/41	3.62	16.1	3.4
6/8/42	4.16	17.0	3.7	1/23/41	4.51	17.6	3.9
9/21/42	4.93	18.3	4.0	4/15/42	4.46	18.5	3.6
9/29/42	4.77	18.3	3.9				
Capillary No. 11							
2/7/47	4.56	18.0	3.8				
2/11/47	3.71	16.9	3.3				

method which he used is sufficiently accurate and whether the results obtained with it necessarily parallel the polarographic findings in disease, where the *lability* of the protein in the alkaline solution may be the determining factor. Also the need for additional equipment for this test might detract from its adoption in clinical work. We are, therefore, contemplating in our further studies the extremely simple copper sulfate technique of Phillips *et al.* (12) for the determination of proteins. Perhaps inclusion of protein data thus obtained may reduce the normal range of the index. *At present we may expect that the protein index*

of a normal individual should be in the range of 1 to 5 and should not vary in repeated tests with the same capillary by more than one unit.

Statistical analysis of all the data in Table I gives us as a mean for the protein index a value of  $2.4 \pm 0.17$  with a standard deviation of 0.95. The frequency distribution is shown in Fig. 2, along with the calculated normal frequency curve.

In curves involving catalytic processes at the dropping mercury electrode the Ilkovič equation cannot be used to compare results ob-

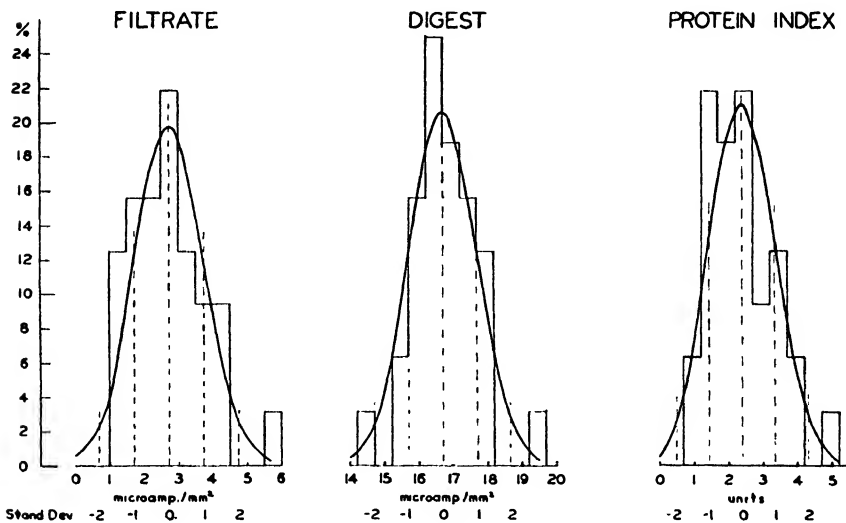


FIG. 2. Frequency distributions and calculated normal frequency curves of filtrate, digest, and protein index values of 32 normal individuals.

tained with different capillaries. We found, however, that the height of the catalytic wave is a function of the surface area of the mercury drops (1). Hence, to permit a comparison of the digest and filtrate wave-heights obtained with the two capillaries, we have evaluated the results in microamperes/mm.<sup>2</sup> of drop surface. For this we have determined the drop weights,  $W$ , in a few typical solutions at an applied voltage of  $-1.6$  v.<sup>5</sup> and calculated the surface area,  $A$ , of the drop as  $0.8517 \times W^{\frac{1}{2}}$  (at  $25^{\circ}\text{C}.$ ). For the abnormal capillary No. 1, we thus

<sup>5</sup> In our first paper (1) we measured the drop weights at  $-0.8$  v.; our present procedure is more correct and assures better agreement.

obtained  $W = 1.42$  mg. and  $A = 1.07$  mm.<sup>2</sup>; for the normal capillary No. 11 the corresponding values were 2.91 mg. and 1.74 mm.<sup>2</sup>

The data thus obtained are listed in the last two columns of Table I. They show good agreement between the two capillaries, and statistical analysis indicated that the difference between the capillaries in both tests was not significant. The mean of all values for the filtrate test is  $2.72 \pm 0.18$   $\mu$ A./mm.<sup>2</sup> with a standard deviation of 1.01; that for the digest test is  $16.7 \pm 0.17$   $\mu$ A./mm.<sup>2</sup> with a standard deviation of 0.97. On the basis of these data we have plotted the frequency distributions and calculated the normal frequency curves shown in Fig. 2.

*Effect of Sex.* Statistical analysis showed that the differences in the mean values of the protein index of males and females was not significant; the difference between the means was less than twice the standard error.

*Effect of Age.* No correlation was noticed when filtrate, digest or index values were plotted vs. age. This finding is in accord with Milam's studies of blood proteins in relation to age (13).

*Effect of Menstruation and Ovulation.* This was studied in five individuals over periods of two months or more. The changes observed were inconsistent and so small that they can be neglected in our evaluation of a range for normal plasmas. The results obtained in two representative cases are shown in Fig. 3.

*Effect of Pregnancy.* Our limited number of observations on pregnant normal individuals, presented in Table III, leads us to expect that the

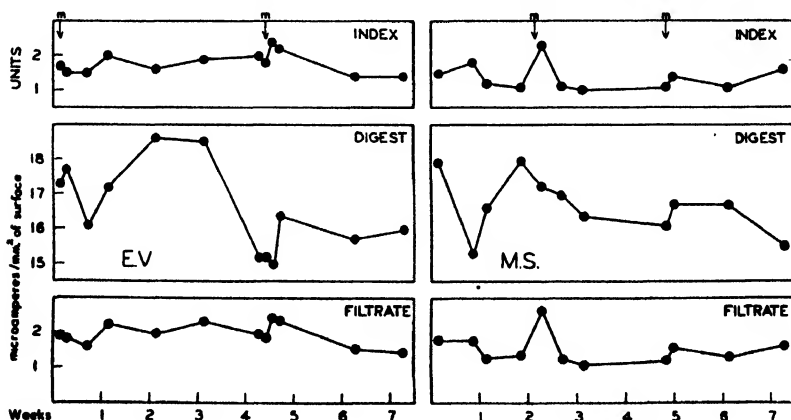


FIG. 3. Effect of menstruation (m) on filtrate, digest, and protein index values.

TABLE III

*Filtrate, Digest and Protein Index Values in Normal Pregnancies*  
(Capillary No. 1)

Subject	Time during pregnancy	Current in $\mu\text{A./mm.}^2$		Protein index
		Filtrate	Digest	
E. M.	Before pregnancy	2.75	17.3	2.4
	Before pregnancy	2.34	15.7	2.2
	2nd month	2.75	16.4	2.5
	6th month	3.30	15.5	3.2
I. M.	14 days a.p.	3.16	12.0	3.9
J. B.	3 days a.p.	2.75	13.9	3.0
C. W.	1 day a.p.	2.40	13.2	2.7
H. K.	1 day a.p.	2.96	11.5	3.9
C. K.	7 hours a.p.	4.54	12.9	5.3

index may rise slightly with the duration of pregnancy but still remain within our normal limits. Larger deviations may occur in the presence of complications, as will be shown in a subsequent publication.

*Normal Protein Index of Several Animals.* No special study was made to determine the normal range for the different animals; the results presented here were obtained incidentally in connection with experiments designed to ascertain the change in the protein index during certain procedures. We feel that the values given are, nevertheless, fairly representative, since alterations in the values could be brought about by experimental procedures which, if not too drastic, were followed by a return to the "normal" level. Furthermore, we found that animals (dogs) fresh from the supply house, with a high protein index but with no outward signs of any disease, would show an index approaching our "normal" levels after suitable care and treatment. If animals with high protein indexes were used in surgical experiments, they showed very poor recoveries, and often died even after relatively simple procedures.

The data for normal animals are summarized in Table IV. Again it is striking to see such a relatively small range and little variation between the different species in the protein index, in spite of the known difference in protein content and the observed differences in the digest and filtrate values.

TABLE IV  
*"Normal" Filtrate, Digest and Protein Index Values of Different Animals*  
 (Capillary No. 1)

Animal		Current in $\mu\text{A./mm.}^2$		Protein index
		Filtrate	Digest	
Monkey	No. M107	3.72	12.1	4.6
	No. M122	5.36	13.1	6.1
	No. M123	4.35	13.6	4.8
Horse	N	1.55	9.8	2.4
	P	1.92	10.2	2.8
	H	3.30	8.3	5.9
	C	2.82	9.7	4.4
Dog	A	3.50	12.0	4.4
	A	2.83	11.3	3.7
	C	4.18	13.2	4.7
	C	4.73	12.5	5.6
Rabbit	A	0.76	15.6	0.7
	B	1.08	14.1	1.2
	C	2.88	13.5	3.2
	D	2.24	16.3	2.1
Rat	No. 5	1.55	10.0	2.3
	No. 9	2.78	10.9	3.8

With respect to the rat, it seemed worth while to report the data shown in Table V. These illustrate how repeated withdrawals of relatively large quantities of blood (by heart puncture) can be withstood by the rat and produce only small changes in the protein index. Micro Kjeldahl determinations of plasma proteins are also reported in this table; the protein content does not seem to parallel the digest values as much as expected (11).

#### ACKNOWLEDGMENTS

We wish to express our appreciation to Mr. D. M. Phillips for technical assistance and to the subjects for their cooperation.

We are indebted to Dr. K. Dobriner for blood samples from monkeys, to Dr. Roy W. Bonsnes for blood samples from pregnant women, and to Dr. John L. Gedgoud for aid in the statistical treatment.

TABLE V  
*Filtrate, Digest, Protein Index and Protein Nitrogen Values  
of Rats after Repeated Bleedings  
(Capillary No. 1)*

Date	Ml. blood drawn	Current in $\mu\text{A./mm.}^2$		Protein index	Plasma protein nitrogen g./100 ml.
		Filtrate	Digest		
Rat No. 9 (310 g.)					
4/23/42	10.5	2.78	10.9	3.8	.745
4/27/42	5.0	3.20	12.2	3.9	.845
4/30/42	3.1	sample ruined			.821
5/5/42	5.0	2.58	10.9	3.5	.695
5/12/42	4.3	2.26	11.7	2.9	.807
5/25/42	5.0	3.24	9.4	5.2	.830
6/8/42	3.5	2.32	10.3	3.4	.761
Rat No. 5 (200 g.)					
5/4/42	5.4	1.55	10.0	2.3	.800
5/5/42	5.0	2.61	10.9	3.6	.695
5/12/42	4.0	1.83	12.2	2.2	
5/25/42	3.5	2.62	11.1	3.6	
6/8/42	4.5	2.25	11.6	2.9	

### SUMMARY

1. Detailed instructions are given for carrying out polarographic filtrate and digest tests on oxalated blood plasma, results of which are used in calculating the protein index. The proposed technique is simple enough to be suitable for routine clinical analyses.

2. The protein index is shown to be remarkably constant for any given individual over long periods of time and to fall within a fairly narrow range for normal men and women even though the experimental conditions may differ considerably. There is no significant difference between the protein indexes of men and women, and there is no appreciable variation in the index with age, or with menstruation and ovulation, and during the progress of normal pregnancies. This constancy of the protein index exists in spite of the fact that the filtrate and digest values may show considerable variations.

3. Index values for different "normal" animals also indicate this constancy and independence of the index from the digest and filtrate values.

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# The Use of Cation Exchangers for the Concentration and Purification of Antibiotics of Basic Nature <sup>1</sup>

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Received May 13, 1947

## INTRODUCTION

While working in the laboratory of Dr. S. A. Waksman, one of us (W. K.) isolated an actinomyces and discovered that it produces an antibiotic which is active predominantly against gram-negative bacteria (1). This actinomyces was subsequently recognized as a strain of *A. lavendulae* and the antibiotic produced by it was named streptothricin by Dr. S. A. Waksman (2, 3). Since the report of the basic nature (2) of the substance, other antibiotics of a basic nature have been described. These substances are streptomycin (4), lavendulin, and actinorubin (5, 6, 7).

During our studies in the purification of the above mentioned basic substances we investigated several possible methods designed to concentrate the antibiotic in question in such a way as to achieve rapidly a fair degree of purity without too great loss of material.

For the purification of streptothricin and streptomycin, adsorption on charcoal and elution with HCl, methanol-HCl or methanol-formic acid is commonly used (2, 4, 8, 9, 10, 11, 12). However, the recoveries obtained by this process are usually in the neighborhood of about 30–60% (10, 11). We have investigated the use of cation exchangers for the concentration and purification of streptothricin, and have found that considerably higher recoveries may be obtained.

<sup>1</sup> This project has been supported by the Thomas Harvey Dougherty, Jr., Fellowship for Research in Brucellosis Fund and by grants from Smith, Kline, and French Laboratories and the Department of Agriculture, Commonwealth of Pennsylvania.

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From the results obtained we are inclined to believe that some of our methods might have general application for the concentration and purification of antibiotics of a basic nature and therefore they are reported here, using streptothricin as an example. Most of the experiments were carried out about 4 years ago.

## EXPERIMENTAL

### *Assay*

The antibiotic activity of streptothricin is assayed by the streak test. Specified amounts of the antibiotic are mixed with 10 ml. of Bacto-nutrient agar pH 7.3 in a petri dish (Bacto-nutrient agar, dehydrated, as used in standard methods of water analysis was used, containing 5 g. Bacto-peptone, 3 g. Bacto-beef extract and 15 g. agar/1000 ml. distilled water. The medium is adjusted so that after autoclaving the reaction will be pH 7.3.) After solidification the test organism (*Escherichia coli*, University of Pennsylvania strain P-216) is streaked upon the agar and the plates are incubated 18–20 hours at 37°C. The smallest amount of the antibiotic per ml. of nutrient agar which will suppress the growth of the test organism is designated one unit. On multiplying our units by the factor 0.25 they compare approximately with the units for streptothricin given by other investigators (8, 9, 10).

### *Production of Streptothricin*

In order to produce a sufficient crop of spores, *A. lavendulae* is grown on dextrose-asparagine agar at 28°C. for 6–10 days. The spores are suspended in saline and the vessels containing the medium to be inoculated are seeded heavily while the medium is still warm (40–45°C.). The cultures are placed in the incubator at 28°C. The vessels usually used for the shallow layer cultivation are Pyrex Roux flasks, containing 300 ml. of the medium, the composition of which is: Glycerin 5 ml., Bacto-tryptone 5 g., NaCl 2 g., K<sub>2</sub>HPO<sub>4</sub> 2 g., FeSO<sub>4</sub>·7H<sub>2</sub>O 10 mg., agar 2 g., distilled water to 1000 ml. Under these conditions the maximum production of antibiotic is reached in 5–8 days of incubation at 28°C., at which time the pH is about 8.0–8.4 and the antibiotic activity assays between 400 and 500 units/ml. After the production of streptothricin has reached its peak, the culture fluid is filtered through large folded filters to remove

TABLE I  
*Production of Streptothricin by Actinomyces lavendulae*

Days	1	2	3	5	6	7	8	12
Units/ml.	—	20*	50	400	500	500	400	400
pH	7.0	6.6	6.9	7.7	7.9	8.2	8.4	8.4

\* Numbers are units of streptothricin/ml. of filtrate of crude culture.

pellicle and agar. At this stage traces of a blue pigment, formed on the underside of the pellicle, can be seen. The light brown color of the crude culture deepens considerably during the filtration. The crude culture filtrate will keep its antibiotic titer for many weeks if stored in the refrigerator either at pH 8 or adjusted with HCl to pH 7. Table I shows the production of streptothricin on the above mentioned medium. Two types of cation exchangers were investigated for the purification of streptothricin, the zeolite and the resinous types.

#### CONCENTRATION OF STREPTOTHRICIN WITH THE AID OF ZEOLITES

Streptothricin is taken up by decalso or permutit and can be released from it by concentrated salt solutions. A typical example will suffice.

Thirty liters of streptothricin crude culture filtrate, assaying 500 units/ml. (15 million units), and having a pH of 8.3, were adjusted with 10% HCl to pH 7.0 and stirred with 600 g. of decalso<sup>3</sup> (50-80 mesh), 20 g./l., for one hour at room temperature. The supernatant, containing almost no activity (5-10 units/ml. at the most), was discarded and the decalso washed with distilled water and dried with acetone on a Buchner funnel. The decalso was spread out and dried at room temperature or at 30°C. until free of acetone odor. The decalso containing the adsorbed streptothricin can be stored in the refrigerator for months without loss of antibacterial potency.

From the dried and acetone-free decalso, streptothricin is eluted with a minimum of saturated solution of NaCl by pouring the decalso in a column of about 3 cm. inside diameter and 120 cm. long (such a column will hold about 450 g. of decalso, 50-80 mesh), tapping the decalso tight and percolating the salt solution through it at effluent rate of about 2 ml./min. until 15 ml./20 g. decalso are collected. The recovery is, under those conditions, 95-98%. Separating the various fractions of the percolate and testing them for antibiotic activity, we found that the first fraction is water clear and inactive, apparently containing mainly displacement water. The next fractions contain the bulk of the pigment and antibiotic activity, while the latter fractions have very low activity (see Table II).

The normal procedure followed was not to elute the antibiotic directly from the column, but to soak the decalso in the column first with the saturated solution of NaCl at a rate of about 2 or 3 minutes/20 g. of decalso, close the column and allow it to stand over night.

<sup>3</sup> Much valuable information, as well as samples of zeolites, has been made available to us through Dr. A. B. Mindler of the Permutit Co., New York, to whom we are grateful.

Next day the solution of NaCl was released from the column at a rate of about 1.5 ml./min. until 15 ml. of eluate for every 20 g. of decalso had been collected. In this way practically all the activity is recovered (98%) and the volume of the eluate is about 1/90th that of the crude culture filtrate. From 450 g. decalso, 340 ml. are collected. The eluate in this state is quite stable, even at room temperature, and can be kept in the refrigerator for months without losing potency.

TABLE II

*Exchange of Streptothricin from Decalso by Saturated NaCl Solution*

Fraction no.	Eluate	Activity	Color <sup>*</sup>
	<i>ml.</i>	<i>units</i>	
1	25	—	colorless
2	150	7,500,000	brown
3	100	3,000,000	yellow
4	50	600,000	light yellow
5	50	100,000	colorless
6	50	—	—

*Note.* A column of 3 cm. inside diameter and of 120 cm. in height was filled with 450 g. of decalso containing a total of 11.2 million units of streptothricin. A saturated solution of NaCl was passed through the column at a rate of 2 ml./min. and the fractions indicated above collected separately and tested for antibiotic activity.

The eluate (340 ml.) is evaporated to dryness at 45°C. *in vacuo* and dried further in the vacuum dessicator over P<sub>2</sub>O<sub>5</sub>. The dried material, containing the streptothricin and a considerable amount of NaCl, is soaked over night at room temperature in 150 ml. of absolute methanol. It is then pulverized, extracted by boiling the methanol about 5 minutes and filtering off from the undissolved NaCl while still hot through a sintered glass funnel. Usually, this filtrate contained 75% of the total activity. A second treatment, carried out as before, with 75 ml. of methanol, extracted an additional 10% of the activity.

The methanol extracts become cloudy upon cooling and, on standing in the cold for a day or longer, deposit a sticky brown inactive residue which usually clings to the walls of the vessel. After removal of this residue the combined methanol extracts assayed about 85% of the total activity. The methanol extracts can be kept in the refrigerator for months without losing potency.

For further purification there is added to the ice cold methanol

extracts half their volume of cold ether with stirring. The resulting thick precipitate is centrifuged in the cold and, after discarding the supernatant, washed twice with 200 ml. of cold absolute ethanol. The precipitate containing the streptothricin is finally dried *in vacuo* over  $P_2O_5$  at room temperature. The treatment with ether results in a loss of 2–4% of the total activity, but removes a considerable amount of brown pigments and inert material. The washings with ethanol result in a loss of only 1–2% of the total activity. The dried material is dissolved in 100 ml. of distilled water and stirred with 0.5 g. of acid-washed charcoal (Norit A) for 30 minutes at room temperature, filtered, and the charcoal washed with several small portions of distilled water, so that the total volume is about 150 ml. This treatment removes all the brown pigment and again some inert material and only 2–5% of the total activity. The resulting material is finally lyophilized. The product thus obtained is a white powder which is very hygroscopic and assays on the average from 1 to 2  $\gamma$ /unit. It contains from 5 to 10% ash (NaCl). The total yield is between 70 and 80% of the original activity. This material represents about 15 to somewhat over 30% pure streptothricin, when compared with the data in the literature given for pure streptothricin (8, 9, 10), and can be used directly for the final steps of isolation by chromatographic adsorption on activated alumina, as shown, for instance, on the purification of streptomycin (10). In addition to the compounds reported forming difficultly-soluble salts with streptothricin (8, 9, 11), the following sulfonic acids<sup>4</sup> and compounds also form difficultly-soluble salts with streptothricin: flavianic acid, azobenzenesulfonic acid, *p,p'*-hydroxyazobenzenesulfonic acid, nitranilic acid and rhodanilic acid.

The zeolite method can be further simplified by carrying out the adsorption process of streptothricin in a column, instead of stirring the material with decalso. We have used in preliminary experiments a glass tube of about 4 cm. inside diameter and about 100 cm. in height. A mixture of equal parts of decalso 50–80 mesh and 10–50 mesh was found suitable. After wetting the decalso with distilled water, 30 l. of crude culture (500 units/ml.) adjusted to pH 7 were percolated through the column at a rate of 30–40 ml./min. The exchange was quantitative. The approximate rate of saturation of the decalso with streptothricin

<sup>4</sup> We are greatly indebted to the late Dr. Max Bergmann of the Rockefeller Institute for Medical Research for repeated valuable suggestions and generous samples of a variety of sulfonic acids.

can be followed by observing the dark band formed during the percolation process. During the hot season we found it advantageous to carry out the column adsorption in the cold room to avoid bacterial contamination.

The advantages of this process are the good yields obtained. Furthermore, the purification may be interrupted after every step, if so desired, and the material stored for months without loss of activity. The small amount of streptothricin not extracted by methanol from the NaCl can be recirculated in the process and higher yields thus obtained.

The disadvantage of this process is the introduction of NaCl as an impurity which is difficult to remove.

We have tried to substitute for NaCl other salts and various compounds in the exchange process, but were unable to find a more suitable eluant than NaCl. Saturated KCl and  $\text{NH}_4\text{Cl}$ , which were suggested (13), work at least as well, but are undesirable because of their ability to form rather insoluble salts with many sulfonic acids which form addition compounds with streptothricin. Many other salts, such as those of Ba, Ca and Mg, were found unsuitable, together with  $(\text{NH}_4)_2\text{CO}_3$ ,  $\text{NaHCO}_3$ ,  $\text{Na}_2\text{CO}_3$ , etc., and some of the aliphatic amines.

#### CONCENTRATION OF STREPTOTHRICIN WITH THE AID OF RESINOUS EXCHANGERS

For some time we have tried to utilize cation exchangers of the resinous type for the concentration and purification of streptothricin. Their great capacity and ease of handling seemed to make them particularly suitable for this purpose.

When a crude culture filtrate of streptothricin, adjusted to pH 7, is passed through a column of amberlite IR-100 (Na-cycle) <sup>5</sup> with the necessary precautions (14), complete exchange of streptothricin takes place. Studying the rates of flow through the amberlite bed we arrived at the following formula for complete adsorption of the antibiotic under

the following conditions: 
$$\frac{\text{Volume IR-100}}{4-5} = \text{ml. flow/min.}$$
 The volume

of the amberlite was measured after swelling. The above formula was found to be quite reliable using columns of various heights and diameters.

<sup>5</sup> For generous samples of different amberlites we are greatly indebted to the Resinous Products and Chemical Co., Philadelphia, Pa., especially to Mr. E. R. Mueller and Dr. J. Winter for many useful suggestions and help.

The adsorption of streptothricin is excellent and sharply defined up to the breakthrough. The capacity of IR-100 for streptothricin is about 4 times greater than that of decalso on a dry weight basis. To give an example, 100 ml. of moist amberlite (Na-cycle) will exchange quantitatively 10 million units of streptothricin contained in 20 l. of a crude culture, assaying 500 units/ml.

However, the removal of the streptothricin from the amberlite is not accomplished as easily as it is from the decalso. Several treatments with saturated KCl were necessary to recover 80% of the adsorbed streptothricin, and strong HCl removed at best only 40–66% of the activity. The highest exchange is accomplished by certain simple primary amines (used as hydrochlorides) having a dissociation constant of  $8 \times 10^{-5}$  or greater, such as propylamine, ethylenediamine and

TABLE III  
*Recovery of Streptothricin from Amberlite IR-100<sup>a</sup> Using Various Eluents*

Eluent used	Volume of eluent	Per cent recovery	Remarks	Dissociation constant
4% HCl	ml. 50	—	at 2°C.	
7% HCl	35	10–25	at 2°C.	
10% HCl	35	40–66	at 2°C.	
8% NaHCO <sub>3</sub>	25	20	at room temperature	
2 M NH <sub>4</sub> Cl	25	40	at room temperature	
Sat. KCl	3×30 <sup>b</sup>	80	at room temperature	
M/2 Guanidine-HCl	30	13	at room temperature	$1.1 \times 10^{-8}$
M/2 Piperidine-HCl	30	13	at room temperature	$1.6 \times 10^{-3}$
2 M Ethylenediamine	25	90–100	at room temperature	$8.5 \times 10^{-5}$
2 M <i>n</i> -Propylamine <sup>c</sup>	25	100	at room temperature	$4.7 \times 10^{-4}$

<sup>a</sup> 5 ml. of amberlite IR-100 which had 100,000 units of streptothricin absorbed were placed in a column of 7 mm. inside diameter and eluted with the above mentioned substances at a rate of 0.2 ml./min.

<sup>b</sup> Three consecutive elutions with 30 ml. of saturated KCl each removed in decreasing order, 47, 25 and 8% of the streptothricin adsorbed on the amberlite (80%).

<sup>c</sup> For samples of different amines, we are indebted to the Sharples Chemical Co., Inc.

others. Incomplete elution was accomplished by guanidine salts,  $\text{Na}_2\text{CO}_3$ , piperidine, *etc.* (see Table III). No, or only traces of, streptothricin was recovered from the amberlite with saturated  $\text{MgSO}_4$ ,  $(\text{NH}_4)_2\text{SO}_4$ , 4% HCl in methanol,  $M/3 \text{ NH}_3$ ,  $M/2 \text{ BaS}$ ,  $M$  nicotine  $M$  morpholine, under the condition of our experiments.

Although quantitative recovery of the streptothricin from the amberlites can be accomplished, as for instance with propylamine, the introduction of another base in the eluate complicates further the purification of the antibiotic. For this and other reasons we preferred for the time being to use decalso rather than the amberlites.

#### NOTES AND COMMENTS

In our opinion the use of cation exchangers for the concentration of antibiotics of a basic nature is practical and economical. We have used it for the concentration and purification of two other antibiotics (5) with satisfactory yields. The use of zeolites for the purification of streptomycin was considered (12) but given up as being too nonspecific.

In going through the process of purification with the zeolites a few additional notes might be in order. Adjusting the crude culture fluid to a pH of 7.0 prior to the exchange on decalso, is important for the complete adsorption of streptothricin. The dried NaCl extract containing the streptothricin can also be extracted with methanol at refrigerator temperature, instead of with boiling methanol. In such manner somewhat purer preparations of streptothricin may be obtained. However, a single extraction with cold methanol will remove not more than 25–40% of the total streptothricin present, using the same amount of methanol. Frequently we extracted only once with boiling methanol, obtaining about 75% of the total activity and recirculated the unextracted NaCl residue in the elution process. The disadvantage of the time-consuming percolation of the crude culture through the decalso can be prevented by carrying out the column adsorption in the cold room over night.

Substances removed by permutit were shown to be relatively strong bases of a dissociation constant of  $5 \times 10^{-8}$  or greater (13). From the chemical point of view the substances taken up by permutit are, in general, alkyl amines and not aryl amines. The recovery of the bases can be accomplished quantitatively by saturated KCl and  $\text{NH}_4\text{Cl}$  solutions.

Similar conditions seem to prevail with the amberlites (14, 15) of which amberlite IR-100 was used for the exchange of streptothricin. However, due to the fact that the active exchange group in the amberlite IR-100 is an  $\text{SO}_3\text{H}$  group, which binds streptothricin rather tenaciously, or to other conditions, the base cannot be recovered with the same ease as from the decalso. For this reason we preferred the zeolites for small scale operation. In large scale operation, however, the great capacity of amberlite IR-100, and the fact that partially eluted amberlite can be used over again, make it quite suitable for continuous operation. While column operation is indicated for the adsorption process, stirring the amberlite for the recovery of the base can be used with results almost as satisfactory as in column operations. The uptake of streptothricin by amberlite was found to be a true exchange and not a surface adsorption.

When 10% HCl is used for the elution of streptothricin from the amberlite, the resulting strongly acid eluate can be neutralized rather quickly by passing it through a column of amberlite IR-4 which will take up the HCl but none of the streptothricin.

### SUMMARY

The use of cation exchangers (decalso and amberlite IR-100) for the concentration and purification of streptothricin was investigated. The yields of streptothricin obtained are higher than with other methods of purification. The feasibility of cation exchangers for the purification of antibiotics of basic nature is suggested.

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# Studies on Carotenoid Metabolism. VII. The Site of Conversion of Carotene to Vitamin A in the Rat <sup>1</sup>

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Received May 27, 1947

## INTRODUCTION

It has been recognized for many years that carotene is converted into vitamin A in the animal body. Generally, this function has been ascribed to the liver, although the evidence on which this conclusion is based is far from satisfactory. That the liver is the organ responsible was originally postulated by Moore (1). Later, Olcott and McCann claimed to have demonstrated such a change *in vitro* (2). Later workers have, in general, been unable to repeat this work (3-4), although successful results were obtained in some cases (5-6).

Earlier work from this laboratory (7) presented the paradoxical situation in which rats injected parenterally with carotene died of vitamin A deficiency in spite of the fact that their livers contained large amounts of carotene. Moreover, after the parenteral administration of carotene, no vitamin A could be found in the livers of the animals. The possibility was suggested at that time that the wall of the intestine might be the site of conversion.

Popper (8), using fluorescent microscopy, was led to somewhat the same conclusion. He ascribed the site of conversion to either the intestine, liver, kidney, lung, renal cortex or adrenal cortex, or to all of these.

A preliminary report of the papers to be presented at the April, 1947, meeting of the Biochemical Society included one which supports the claim that, in the rat, carotene is converted to vitamin A in the intestinal wall (9). The paucity of information which can be included in such a report makes evaluation of the results difficult, although it would seem that our approach to the problem has been somewhat different.

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<sup>1</sup> This investigation was made possible through a grant from the Nutrition Foundation, Inc. Some of the data reported here were presented at the 28th annual meeting of the Pacific division of the A.A.A.S. at San Diego, California, June 16-21, 1947.

<sup>2</sup> These data are to be presented in partial fulfillment of the degree of Doctor of Philosophy in the Graduate School of the University of Southern California.

In the present paper, quantitative experimental evidence is offered which indicates that one site of conversion of carotene to vitamin A in the rat is the intestinal wall.

### METHODS

The animals used were from our stock colony. The dietary regime was such as to make the animals suitable for use in the biological assay for vitamin A. The parents were maintained for at least two months previous to breeding and for 14 days after birth of the litter on Sherman diet B (10) without the addition of supplementary lettuce or meat. Litters were reduced to 7 at 3 days of age, and on the 14th day mothers and litters were placed on a vitamin A-low diet.<sup>3</sup> The animals were weaned at 21 days and placed on the USP XII deficient diet, on which they were maintained throughout the duration of the experiment. After 3 weeks on this last diet the animals were suitable for use, in that no vitamin A was found on analyses of various tissues. The animals supplemented with cottonseed oil in the following experiments offer proof of this. Any animals that showed a marked drop in weight or xerophthalmia were discarded as unfit for use.

The carotene solution used was prepared by diluting Valley Carotene<sup>4</sup> with cottonseed oil containing 0.5% tocopherol so that the final concentration of carotenoid was 1800  $\gamma$ /ml. A comparison of the potency of this preparation by the biological and spectrophotometric methods with crystalline  $\beta$ -carotene indicated that they were approximately identical. Apparently the method of manufacture removes most of the carotenoids other than  $\beta$ -carotene normally found in alfalfa. In one series of experiments the same cottonseed oil without added carotene was used. These two supplements were administered by stomach tube. In the vitamin A feeding experiment a fish liver oil concentrate containing 130,000 I.U./g. was used. This was administered *per os*.

For the analysis of the intestinal tract, the entire tract was removed and approximately the upper two-thirds of the small intestine was separated. This portion was used for analysis after flushing out the contents with 100 ml. of 0.9% aqueous sodium chloride. The entire liver was removed, and after removal of the adhering blood by blotting, this organ was cut into small pieces. The two kidneys were removed, any adhering connective tissue and the kidney capsule were discarded, and the kidneys combined into one sample after being cut into small pieces.

The samples were saponified for 30 minutes, using 20 ml. of 95% ethanol as a solvent and one ml. of 50% aqueous KOH/g. of tissue. The cooled saponified mixture was then extracted with Skellysolve A. The extract was washed with water and dried with anhydrous sodium sulfate. The E450 value was determined in the Coleman Model 6A Junior Spectrophotometer. The sample was then evaporated to dryness in a stream of nitrogen and the residue taken up in 1 ml. of chloroform. Vitamin A was determined by the Carr-Price reaction using 5 ml. of a saturated solution of  $\text{SbCl}_5$  in

<sup>3</sup> The same as the USP XII vitamin A-deficient diet, except that commercial casein is used in place of alcohol-extracted casein.

<sup>4</sup> This was obtained through the courtesy of Dr. Francis P. Griffiths of the Valley Vitamins, Inc., McCallen, Texas. It contained 22,400  $\gamma$ /g.

chloroform. One drop of acetic anhydride was first added, to preclude the development of a cloudy solution. The E620 value was corrected for the carotene increment in the case of the intestinal tract but not for the liver. The reason for this difference will be apparent from the rest of this paper. The instrument was standardized and checked at frequent intervals using a mixture of 90%  $\beta$ - and 10%  $\gamma$ -carotene <sup>5</sup> and distilled Vitamin A Ester.<sup>6</sup>

In the experiment where an attempt was made to identify the yellow pigment in the liver, the extraction was carried out with freshly redistilled diethyl ether. The absorption curve was determined on the Beckman spectrophotometer.

All determinations were carried out in a darkened room equipped with a special lighting system to minimize destruction of vitamin A by light.

## RESULTS

### *A. The Effect of the Administration of Cottonseed Oil*

The vitamin A-depleted animals were stomach tubed with 0.5 ml. of cottonseed oil containing 0.5% tocopherol. The animals were killed at varying intervals and analyses of the livers and intestinal tracts carried out. Results are given in Table I.

These animals, although they have never had access to any carotenoid pigment (except that contained in the milk obtained during nursing), have a chromagen in the liver which absorbs light at 450 m $\mu$ .

TABLE I

*The "Carotene" and "Vitamin A" Content of the Liver and Intestinal Tracts of Vitamin A-Depleted Rats at Various Periods after Stomach Tubing with Cottonseed Oil*

Tissue examined	Time elapsed in hours					Average
	1	2	4	5	6	
Total apparent carotene in $\gamma$						
Liver	2.60	2.08	1.60	1.00	2.72	2.00
Intestinal tract	1.80	1.20	1.60	1.28	0.72	1.32
Apparent Vitamin A in I.U./g.						
Liver	1.53	1.75	1.52	0.80	2.69	1.66
Intestinal tract	1.06	1.19	2.15	1.17	0.91	1.30

<sup>5</sup> Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

<sup>6</sup> Obtained from Distillation Products, Rochester, New York.

For purposes of comparison the values are reported as if they were carotene using the factor of  $E_{1\text{cm}}^{1\%} = 2500$ .

Similarly, although values are reported for vitamin A, we do not believe them to be due to the vitamin. Identical results have been obtained on animals in the last stage of vitamin A deficiency. The apparent vitamin A value of 620  $\text{m}\mu$  is due to an amber color which develops immediately on adding the Carr-Price reagent. This color remains constant for 1-3 minutes and then gradually increases in intensity. It would be possible to correct the vitamin A values obtained in the following experiments for this reading. However, we consider it preferable to report the values obtained. If, on adding the Carr-Price reagent, a fading blue color did not develop, this fact is indicated by enclosing the values obtained in parentheses. Such values, although reported as International Units, are not believed actually to represent vitamin A. Since this non-specific color is shared approximately equally by both the liver and the intestine, it does not alter the comparative results.

### *B. The Disappearance of Vitamin A from Various Organs*

In this experiment a group of vitamin A-depleted animals was supplemented *per os* with a single dose of 0.1 ml. of the fish liver oil concentrate containing approximately 12,000 I.U. The animals were then continued on the vitamin A-deficient diet. At various intervals, animals of this group were sacrificed and the vitamin A content of the various tissues determined. These results are given in Table II.

Here we see that vitamin A is present in the intestinal wall in considerable amounts for approximately 7 days after the feeding of vitamin A. At the end of this time, it disappears entirely. The only exception to this was one animal where an appreciable quantity still remained in the intestinal wall on the 14th day. In the case of the kidney a different situation obtains. Here vitamin A was present throughout the period studied. These experiments were carried out to determine whether the presence of a fairly high level of vitamin A in the liver would, of itself, lead to the presence of appreciable vitamin A in the intestinal wall. It is clear that, even with levels of 600 to 800 I.U./g. in the liver, sufficient vitamin A is not carried to the intestine to increase the levels in the latter beyond the apparent values found in depleted animals.

TABLE II

*The Vitamin A Content of Various Tissues of Rats Supplemented with a Single Dose of High Potency Fish-Liver Oil Concentrate and then Maintained on a Vitamin A-Free Diet*

Elapsed period	Liver	G. I. tract	Kidneys
<i>days</i>	<i>I.U./g.</i>	<i>I.U./g.</i>	<i>I.U./g.</i>
1	500	25.2	
3	907	10.3	
7	985	19.7	5.97
7	832	14.6	18.0
9	674	(2.4)	
11	880	2.8	
13	874	(2.3)	
14	623	(3.1)	7.57
14	1030	7.97	5.37
17	677	(4.42)	8.50
17	842	(3.43)	6.90
20	738	(1.07)	6.27
20	603		7.68
23	653		8.32
23	952		9.64

*C. The Rate of Appearance of Vitamin A in the Intestinal Tract and the Liver after Feeding Carotene*

In this series vitamin A-depleted animals were stomach tubed with 0.5 ml. of the carotene solution containing approximately 900  $\gamma$  of carotene. After varying periods, the animals were sacrificed and the liver and intestinal tract analyzed for carotene and vitamin A. These results are given in Table III.

Under the experimental conditions employed, vitamin A appeared in the intestinal wall 1.5 hours after feeding carotene, whereas 2.75 hours elapsed before it appeared in the liver. Until approximately 4 hours after feeding, the values in the intestine exceed those in the liver.

The values for the yellow pigment in the liver showed no significant or consistent increase at any time during the period studied or when compared to the values found in the earlier experiments after feeding cottonseed oil. However, this does not preclude entirely the presence of small amounts of carotene.

TABLE III

*The Carotene and Vitamin A Content of the Livers and Intestinal Tracts of Vitamin A-Depleted Animals at Various Intervals after Stomach Tubing with a Solution of Carotene in Oil*

Elapsed period	Total liver carotene	Total intestinal tract carotene	Liver vitamin A		Intestinal tract vitamin A		$\Delta$ Vitamin A <sup>1</sup>
			Total	Per g.	Total	Per g.	
<i>Hrs.</i>	$\gamma$	$\gamma$	<i>I.U.</i>	<i>I.U.</i>	<i>I.U.</i>	<i>I.U.</i>	<i>I.U.</i>
1.5	1.28	3.92	(6.13)	(1.82)	6.67	2.38	+0.56
2.0	1.00	1.00	(5.82)	(2.16)	6.03	3.37	+1.21
2.5	0.72	3.60	(6.13)	(1.92)	8.57	3.89	+1.97
2.75	1.80	10.5	12.7	2.85	15.3	5.14	+2.29
3.0	1.88	13.1	15.9	3.62	26.4	6.02	+2.40
3.0	1.60	13.5	22.8	5.48	40.3	11.1	+5.6
3.0	2.32	14.7	27.5	7.23	36.8	11.1	+3.9
3.25	0.88	3.50	8.89	2.70	9.63	4.13	+1.43
3.50	1.88	6.28	28.6	6.63	22.5	5.72	-0.91
3.75	1.92	32.8	27.8	6.96	31.5	8.57	+1.61
4.0	1.52	7.28	14.6	3.81	16.2	5.68	+1.87
4.5	1.80	6.48	30.7	5.67	27.2	5.32	-0.35
4.75	1.40	23.8	33.9	7.68	30.0	6.37	-1.31
5.25	1.12	5.56	30.7	6.02	15.9	2.74	-3.28
5.75	2.10	13.9	39.1	7.97	30.2	5.48	-2.49

<sup>1</sup> (I.U./g. intestinal tract) - (I.U./g. liver) =  $\Delta$  vitamin A.

#### *D. The Nature of the Liver Pigment*

Twenty-five vitamin A-deficient animals were stomach tubed with 1 ml. of the carotene solution containing approximately 1800  $\gamma$  of this carotenoid. Seven hours later the animals were sacrificed, the livers pooled and the pigment extracted as previously described. Calculating the quantity of pigment present on the basis of its reading at 450  $m\mu$  as if it were  $\beta$ -carotene gave a value of 63.7  $\gamma$ . On a weight basis, this amounts to 0.4  $\gamma$ /g. This is approximately the same value as that obtained either after feeding cottonseed oil or carotene in the previous experiments.

The absorption curve gave no indication of the presence of carotene. The maximum value was in the ultraviolet range and the readings fell linearly from 320 to 520  $m\mu$  which was the region of the spectrum studied. No inflection in the curve was observed at 450  $m\mu$ . The pig-

ment was found to be precipitated from a methyl alcohol solution when placed in a dry ice bath. It was not precipitated from 1:1 acetone-alcohol by digitonin. Although the proof presented here is not absolute, at least the absence of a carotenoid pigment seems to be indicated.

### DISCUSSION

The experiment in which the vitamin A level was followed after feeding carotene demonstrates that vitamin A appears first in the intestinal wall and only later in the liver. This excess of vitamin A in the intestinal wall over that in the liver continued for approximately 4 hours. If one is to accept the liver as the site of conversion, it is difficult to explain how this situation could be brought about.

Even the qualitative presence of vitamin A under these circumstances takes on added significance in view of the fact that it was shown that, following vitamin A supplementation, the vitamin is absent from the intestinal wall even when large amounts are present in the liver. This would indicate that the source of the vitamin A in the intestinal wall is not the liver.

That the liver is not the site of conversion is further indicated by the lack of any increase in its content of carotene, whereas that of the intestinal wall does increase following the feeding of carotene.

The failure of the liver to show an increase in yellow pigment is in disagreement with the work of Moore (1), but in agreement with Sexton *et al.* (7). However, the conditions of Moore's experiment were different from ours, in that his rats were fed large amounts of carrots or red palm oil for several weeks. Under such circumstances Moore showed an increase in the "yellow units" of the livers but the pigment responsible was not identified. Since the supplement he used contains a mixture of carotenoids, it is possible that the pigment was not one of the provitamins A.

The conclusion that the pigment found in the liver under the experimental conditions which we employed is not carotene is in agreement with the work of Johnson and Baumann (11). They report that the "carotene" measured in the livers and kidneys of their animals was extraneous yellow pigment unrelated to the carotene of the diet since the amounts were the same whether carotene was fed or not. Furthermore, the pigment could be separated quantitatively from added carotene on a chromatogram.



Since the intestine is the only organ of the rat in which an increase in carotene can be found following its administration, and since vitamin A is found in the intestinal wall only in a period following the feeding of carotene or vitamin A, it would appear that the site of conversion of carotene to vitamin A in the rat is the intestinal wall. After the vitamin has been formed by the intestine following the feeding of carotene, the liver acts as a storage depot.

If, as we believe, the intestinal wall is the site of conversion, it is necessary to explain how carotene can be utilized as vitamin A following parenteral administration. Very limited utilization is indicated by the work of Sexton *et al.* (7) and more effective utilization by Tomarelli *et al.* (12). Under such circumstances it could well be that the carotene is excreted into the lumen of the intestine, carried there by either the blood or in the bile. That such a mechanism is possible is suggested by the work of Sexton, in which he showed that, following the intraperitoneal administration of carotené, 5.5% could be recovered in the gastro-intestinal tract and feces. The final step in this scheme would then be the reabsorption of the carotene, at which time it would be converted to vitamin A.

#### SUMMARY

1. Animals fed a single dose of vitamin A, and then maintained on a vitamin A-deficient diet for approximately two weeks, show no vitamin A in the intestinal wall, even though large amounts are present in the liver and appreciable amounts in the kidney.

2. Following the administration of carotene to vitamin A-deficient rats, the vitamin appears first in the intestinal wall. For a period of approximately 4 hours the quantity in the intestinal wall exceeds that in the liver.

3. Following the administration of carotene under the experimental conditions employed, there is no increase in the yellow pigment of the liver whereas there is in the intestinal wall. The pigment of the liver apparently is not carotene.

4. It is, therefore, concluded that the site of conversion of carotene to vitamin A in the rat is the intestine wall.

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# Studies on Carotenoid Metabolism. VIII. The *in vitro* Conversion of Carotene to Vitamin A in the Intestine of the Rat <sup>1</sup>

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Received May 27, 1947

## INTRODUCTION

Experimental evidence recently obtained in our laboratory by Mattson *et al.* (1) and given in the preceding paper has shown that the intestine of rats administered carotene by stomach tube and sacrificed up to 6 hours later contained definite amounts of vitamin A. It seems probable that such a conversion might be demonstrated more effectively *in vitro* since, in the intact animal, vitamin A would be removed about as quickly as formed.

The site of conversion of carotene to vitamin A in the rat was reported to be the liver by Olcott and McCann (2) on the basis of *in vitro* experiments which Sexton (3) was unable to confirm. Later workers repeated these experiments on the livers of a variety of animals. Negative results were reported on shark liver by Euler and Euler (4), on cat liver by Rea and Drummond (5) as well as by Drummond and McWalter (6). Pariente and Ralli (7) obtained one positive test out of 4 experiments on dog livers, while Euler and Klussman (8) demonstrated positive results on cow liver, and Wilson *et al.* (9) on rabbit liver. It would seem quite probable, as pointed out by Zechmeister (10), that the site of this transformation shows a decided species difference. A recent preliminary report of Glover *et al.* (11) has indi-

<sup>1</sup> This investigation was made possible through a grant from the Nutrition Foundation, Inc.

<sup>2</sup> These data are to be presented by Catherine E. Wiese in partial fulfillment of the degree of Master of Science in Biochemistry in the Graduate School of the University of Southern California.

cated the possibility of the conversion of carotene to vitamin A in the intestine but the details of such experiments are not at this time available.

## METHODS

The rats used in this experiment were from our stock colony and were depleted of vitamin A as described by Mattson *et al.* (1). The animals were stomach tubed with one ml. of carotene solution and sacrificed immediately. The carotene concentration was 310  $\gamma$ /ml. and the solution was stabilized with "Tween" <sup>3</sup> containing 0.5% of  $\alpha$ -tocopherol.

The carotene was immediately washed from the stomach into the intestine with Ringer-Locke solution<sup>4</sup> and the intestine removed after ligation at the pylorus and ileocaecal valves. The intestines were incubated in Ringer-Locke solution under anaerobic conditions for 3 hours at 37°C.; they were then removed from the bath, the contents flushed out with 0.9% saline and the washings discarded. The intestinal wall was saponified, extracted and the vitamin A determined by the Carr-Price reaction. The procedures were similar to those described by Mattson *et al.* (1).

TABLE I

*The Carotene and Vitamin A Obtained by Extraction of the Intestinal Tract of Vitamin A-Deficient Rats Isolated Immediately after Carotene Administration and Incubated Anaerobically for 3 Hours*

Rat No.	Carotene	Vitamin A	Rat No.	Carotene	Vitamin A
	$\gamma$	I.U.		$\gamma$	I.U.
10	30.0	25.2	22	11.6	3.2
11	22.2	5.8	23	9.4	3.7
13	31.6	18.0	24	8.8	4.2
14	18.0	8.4	25	17.6	7.4
15	18.4	14.0	26	20.8	9.1
16	5.8	4.0	27	18.2	7.8
17	17.7	9.4	28	36.0	11.1
18	4.7	4.0	29	26.4	5.2
20	12.8	12.6	30	20.0	4.3

In order to prove that the vitamin A determined in the intestinal wall after carotene administration was the result of transformation of this carotenoid, a number of control tests were made. These consisted of incubation of the gut without carotene but with the solvent, analysis of intestine of vitamin A-deficient rats without incuba-

<sup>3</sup> "Tween" 80 obtained from the Atlas Powder Co., Wilmington, Del., was used.

<sup>4</sup> The Ringer-Locke solution employed had the following composition: NaCl, 0.9%; KCl, 0.042%; CaCl<sub>2</sub>, 0.024%; NaHCO<sub>3</sub>, 0.05%; MgCl<sub>2</sub>, 0.02% and glucose, 0.05%.

tion and without carotene, or where carotene was administered and flushed out immediately. Studies were also made on the effect of incubation of carotene with Ringer-Locke solution in the absence of intestine as well as the composition of the Ringer-Locke solution obtained after incubation of the intestines.

## RESULTS

Eighteen experiments were carried out where the transformation of carotene to vitamin A was determined in the intestine of vitamin A-deficient rats incubated in Ringer-Locke solution for 3 hours at 37°C. These results are summarized in Table I while the control experiments

TABLE II

*Summary of the Results Obtained for Vitamin A and Carotene in Extracts of Intestines or Ringer-Locke Solution Unincubated or Incubated Anerobically for 3 Hours*

Group No.	Skellysolve A extracts obtained by following experimental procedures	Number of expts.	Carotene from gut wall or tissue bath	Vitamin A from gut wall or tissue bath <sup>1</sup>
I	Intestines of vitamin A-deficient rats, unincubated	2	$\gamma$ 0	I.U. (3.5)
II	Intestines of vitamin A-deficient rats, incubated with "Tween" and $\alpha$ -tocopherol in lumen	3	0	0
III	Intestines of vitamin A-deficient rats administered carotene but immediately flushed with saline solution	2	9.6	(3.0)
IV	Carotene incubated with Ringer-Locke solution (no intestine)	3	12.7	(2.3)
V	Intestines of vitamin A-deficient rats, incubated with carotene solution	18	18.3	8.7
VI	Tissue bath for V	2	8.0	(1.2)

<sup>1</sup> The values in parentheses are for those tests where no fading blue color was observed. These values do not represent vitamin A.

and the average results of the individual tests from Table I are given in Table II.

### DISCUSSION

In all cases where the intestines were incubated with carotene, some vitamin A was formed. Although these varied from a maximum value of 25.2 to a minimum one of 3.2 I.U. of vitamin per intestine (average 8.7), there was in all cases a blue color which faded rapidly on standing. On the other hand, in the control tests reported here as well as by Mattson *et al.* (1), the calculated values averaged about 4 I.U. (maximum 6 I.U.). However, these did not show the color characteristic of vitamin A but were amber and did not fade on standing.

A possible explanation for the low results on vitamin A obtained in several experiments is the poor absorption of carotene. It is noteworthy that the tests where the vitamin A was the lowest (rats no. 16, 18, 22, 23, 24) were also the tests where the lowest carotene was found in the intestine after incubation. This non-absorption of carotene may be the result of injury to the tissue caused by handling or due to a poor nutritional state of the animal from which the preparation was obtained.

The question was raised concerning the continued activity of the intestine in the absence of oxygen. To test this, the anaerobic glycolysis was determined on slices of the intestine from vitamin A-deficient rats by the Warburg technic using the same tissue bath as employed in our other experiments. After 3 hours the tissue still showed activity. The average  $Q_{O_2}^{N_2}$  was 1.09.

### SUMMARY

The *in vitro* transformation of carotene to vitamin A has been demonstrated to take place in the small intestine of vitamin A-deficient rats under anaerobic conditions.

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# Copper Deficiency in Rabbits. Achromotrichia, Alopecia and Dermatosis

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Received June 2, 1947

## INTRODUCTION

In previous studies of iron- and copper-deficiency anemias in rabbits (15) it was noted that a number of rabbits fed a diet which was very low in both iron and copper became gray and lost a variable amount of hair. In a series of preliminary experiments several vitamins and minerals were added to the deficient diet to see if the achromotrichia could be prevented. It was found that copper added to the diet both prevented and cured the condition. To confirm and extend this observation, two critical experiments were conducted and the results are reported here.

A deficiency of several nutrients has been shown to be associated with a depigmentation and a loss of hair. A recent review of this literature is to be found in the article by Rothman and Felscher (11). The literature indicates that in most cases one of the B vitamins is involved. Less frequently a mineral element has been associated with these symptoms. Gorter (6) reported that a deficiency of copper produced a graying of the hair of black rats. In a later publication Gorter (5) confirmed his previous observations with rats and found that cats developed both depigmentation and baldness when fed a copper-deficient diet. This syndrome was, to a certain extent, corrected by feeding copper and zinc but not by either metal alone. More recently Free (4) found that black rats fed a milk diet became gray. A mineral mixture of iron, copper and manganese prevented and cured this grayness. It was reported that attempts to find which mineral of the three was effective were not conclusive. Keil and Nelson (8) noted that black rats fed a milk diet became gray. The feeding of iron salts did not restore the original color but the addition of copper salts did in about 2 months.

Insofar as the authors are aware, there are no reports associating a copper deficiency with achromotrichia in rabbits. Brouwer (2) in a

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report of milk anemia in rabbits mentioned that rabbits, in addition to developing an anemia, loss of weight and deformities of the leg bones, suffered a loss of hair. That copper might have been involved was not mentioned.

### EXPERIMENTAL

The rabbits used in these studies were black and white Dutch rabbits bred in our laboratory. As soon as the young rabbits were two weeks of age all natural feeds were removed from their cages and they were permitted to consume only their dams' milk and whole milk powder (Borden's Klim) until they were weaned and placed on experiment. The lactating dams were removed to another cage daily and permitted to consume a stock ration.



FIG. 1. A picture of a normal control rabbit.

In the first of the two experiments herein reported, 14 weanling rabbits were divided at random (Tippett's tables) into two equal groups. One group (experimental) received the basal diet of whole milk powder *ad libitum*, and a daily supplement of 2 mg. iron, 1 mg. manganese and 1 drop of percomorph oil. The second or control group received, in addition, 0.4 mg. of copper per rabbit per day. This experiment was continued until the rabbits were 15 weeks of age, at which time they were sacrificed and autopsied. In the second experiment, 32 weanling rabbits were divided at random into two equal groups. The experimental group was fed whole milk powder and a daily supplement of 4 mg. of iron, 1 mg. of manganese and 1 drop of percomorph oil. The control group received an additional supplement of 0.4 mg. of copper. This

experiment was continued until the rabbits were 20 weeks of age, when they were sacrificed and autopsied. All rabbits had free access to distilled water.

Considerable care was taken to keep copper contamination of the basal diet at a minimum. The copper-free iron supplement was prepared from carbonyl iron and is described in more detail in a previous publication (14). Manganese was fed as manganous chloride c.p. and copper (control groups) as cupric chloride prepared from electrolytic copper foil. The fluid milk used as a carrier of the daily supplement was secured from the Cornell University herd of cows and milked directly by hand into clean pyrex glass jars.

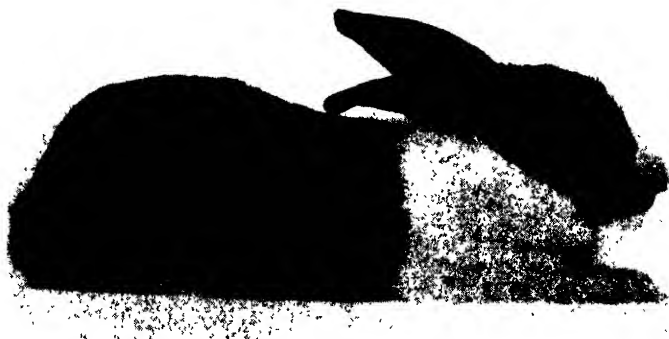


FIG. 2. A copper-deficient rabbit showing extensive graying of the hair.

Hemoglobin was determined as oxyhemoglobin in a photoelectric colorimeter (12). The copper concentration of the liver and blood serum samples was determined by the sodium diethyldithiocarbamate method (3; 10) after the liver samples were dry-ashed at 450°C. and the blood serums were wet-ashed with a mixture of sulphuric, perchloric and nitric acids (100 ml. sulphuric, 50 ml. 60% perchloric, and 50 ml. of a 1:1 re-distilled nitric acid).

During the experiment the rabbits were individually caged in new galvanized screen-bottomed cages. The milk and supplements were fed in pyrex glass custard cups. All animals were weighed weekly and carefully observed for abnormalities.

## RESULTS

A summary of the observed results for both experiments is presented in Tables I and II. The high incidence of achromotrichia, alopecia and

TABLE I

*Summary of Observations on the Hair and Skin*

Treatment	No. of rabbits	Deaths	Achromotrichia	Alopecia	Dermatosis
First Experiment					
+Cu	7	0	0	0	0
-Cu	7	2	5	2	2
Second Experiment					
+Cu	18	1	0	0	0
-Cu	18	5	14	10	9

TABLE II

*Summary of Growth Rate, Hemoglobin, and Copper Concentration in the Liver and Blood Sera*

Treatment	No. of rabbits	Final weight	Final hemoglobin level	Copper concentration in	
				Liver	Blood sera
		(g.)	(g./100 ml.)	( $\gamma$ /g/d.m.)	( $\gamma$ /ml.)
First Experiment					
+Cu	7	1374 $\pm 56.8$	13.0 $\pm 0.52$		
-Cu	5	1172 $\pm 30.2^*$	12.8 $\pm 0.75$		
Second Experiment					
+Cu	17	1477 $\pm 44.7$	12.3 $\pm 0.62$	12.1 $\pm 1.90$	1.63 $\pm 0.240$
-Cu	13	1362 $\pm 54.3^{**}$	10.1 $\pm 0.64^*$	7.2 $\pm 0.57^*$	0.35 $\pm 0.042^{**}$

The above figures are means with their standard errors.

\* Difference significant at odds of 19:1.

\*\* Difference significant at odds of 99:1.

dermatosis among those rabbits fed the basal copper-deficient diet is in marked contrast to the absence of such symptoms in those rabbits supplemented with copper salts. The hair started to turn gray, usually at the base of the tail and on the abdomen, in 4-6 weeks. The graying progressed up the sides and over the back and finally involved the face and ears. At about the same time that graying started, the abdominal hair began to fall out and this progressed to a generalized loss of hair as pictured in Fig. 3. In most, but not all, cases, the bare skin became



FIG. 3. A copper-deficient rabbit exhibiting a marked alopecia.

encrusted with dry, flaky scales of exfoliated epidermis. Such dermatosis showed no gross sign of inflammation.

In the first experiment there was no significant difference between the hemoglobin concentration of the blood of the rabbits fed the copper-deficient diet and those fed copper salts. In previous studies (15) we have readily secured an anemia in rabbits and rats fed a milk diet deficient in both iron and copper. Such anemic rabbits did not give a hemoglobin response when fed pure iron salts alone, which indicated that the iron salts which we were using were essentially free of copper. The second experiment was set up particularly to observe the levels of

hemoglobin between the two groups. In this experiment the intake of iron was doubled over that used in the first experiment and rigid care was taken to hold copper contamination at a minimum. As summarized in Table II, the rabbits fed the basal copper-deficient diet did develop a pronounced anemia. The range of hemoglobin concentrations in the rabbits fed the basal diet was 4.6–13.2 and in the rabbits fed copper salts 10.4–15.3 g. hemoglobin/100 ml. of blood.

The rate of growth of rabbits fed the copper-deficient diet was significantly less than the rate of growth of the copper-supplemented rabbits in both experiments. Also, the concentration of copper in the



FIG. 4. A copper-deficient rabbit illustrating the dermatosis observed.

liver and blood serum was significantly lower in the copper-deficient group, the difference being more marked in the blood serum than in the liver.

Gross sections of the brains of the copper-deficient rabbits were carefully studied but no evidence of lesions as reported for copper deficient lambs (7) was noted.

No detailed studies have been made of regeneration of hair or pigmentation of the copper-deficient rabbits. However, in a few cases where copper salts were fed to such rabbits, there was a complete regeneration of hair growth and normal pigmentation.

## DISCUSSION

The studies here reported show that, in addition to an anemia, a deficiency of copper in rabbits leads to a graying of the hair, loss of hair and a dermatosis. The rat is similar to the rabbit in that it too develops an achromotrichia when dietary copper is limiting, but rats have not been reported to develop an alopecia and dermatosis under similar conditions. The limited observations (2 cats) of Gorter (5) indicate that the cat also develops an alopecia when fed copper-deficient diets.

It appears, particularly from the observations of the first experiment, that the syndrome of achromotrichia, alopecia and dermatosis may be a more sensitive indication of copper deficiency in the rabbit than a decrease in hemoglobin, since this syndrome was more readily obtained than the anemia.

That copper malnutrition might be expected to affect hair pigmentation and structure is suggested by various reports in the literature. Tyrosinase, the oxidizing enzyme thought to be concerned in melanin formation, is a copper proteinate (9). Sarata (13) reported that the amount of copper extractable from mammalian hair is directly proportional to the degree of pigmentation. Bennetts (1) reported that "stringy" wool appears to be the earliest, and frequently the most obvious, sign of copper deficiency in lambs.

## ACKNOWLEDGMENT

The authors are grateful for the technical help of E. M. Gates.

## SUMMARY

In addition to an anemia, copper deficiency in the rabbit results in an achromotrichia, alopecia and dermatosis.

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# Isolation of Rutin from Tomatin Concentrates

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Received June 2, 1947

## INTRODUCTION

In attempts to purify tomatin, an antibiotic agent that occurs in the tomato plant (1, 2, 3), by chromatographic methods, crystalline rutin has been isolated from certain fractions possessing high tomatin activity. It has been shown, however, that rutin does not exhibit antibiotic properties when tested against the fungus, *Fusarium oxysporum* f. *lycopersici* (R-5-6), which is used as the assay organism for tomatin. On the other hand, rutin is known to antagonize the bacteriostatic activity of dicoumarol toward *Staphylococcus aureus* (4).

Rutin (3,5,7,3',4'-pentahydroxyflavone-3-rutinoside) is recognized as one of the most widely distributed glycosides, since it has been isolated from 39 species of plants. Nevertheless, the only report of the isolation of this glycoside from the tomato plant is that of Blount (5) who, by the rather ingenious method of carefully wiping the stems of tomato plants with a linen cloth, collected the yellow exudate formed on the stems, and from it obtained crystalline rutin. At the Eastern Regional Research Laboratory attempts to isolate rutin from fresh green tomato leaves, stems and green fruit of commercial tomato varieties by the customary methods have been unsuccessful.

In the work reported here rutin has been isolated from the dried leaves of the Red Currant tomato plant (*Lycopersicon pimpinellifolium*) This tomato variety, which produces currant-sized fruit, is almost immune to the disease known as Fusarium wilt and for this reason it

has assumed considerable importance in the development of wilt-resistant tomato varieties.

## EXPERIMENTAL

### *Material*

Red Currant tomato vines, grown to maturity in the field at the Plant Industry Station, Beltsville, Md., were harvested and transported by truck to the Eastern Regional Research Laboratory for drying. Approximately 24 hours elapsed between harvesting and drying, which was accomplished by feeding the chopped vines into a forced draft continuous dryer operating at 105°C. and regulated to discharge the dried material one-half hour after entry. The dry leaves and stems were separated in an air separator and the leaves were then ground to pass a 20-mesh sieve. The ground leaves were used for tomatin and rutin investigations.

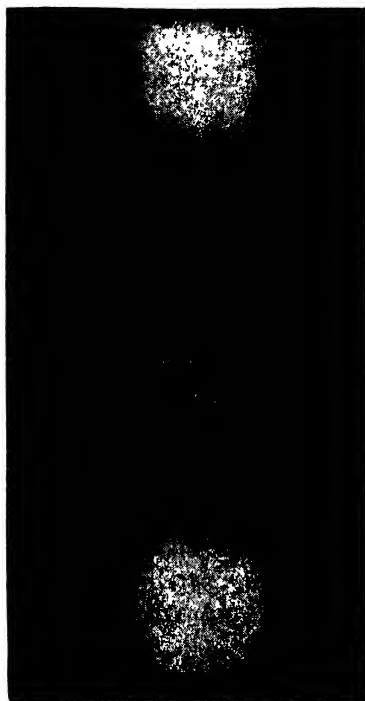


FIG. 1. Chromatographic fractionation of a tomatin concentrate. (Potato starch column; solvent, Butanol-water.) Tomatin and rutin are present in the leading zone.

### Procedure

Dried Red Currant tomato leaves (600 g.) were extracted with hot 95% methanol until most of the green color was removed. The methanol extract was concentrated under reduced pressure to a thick syrup, diluted with 1 liter of water, mixed and autoclaved at 15 lbs. steam pressure for one-half hour. After autoclaving, the solution was allowed to cool to permit most of the chlorophyll, gums and lipids to settle to the bottom of the flask, and the supernatant solution was decanted, centrifuged and concentrated to 50 ml. (The tomatin activity, as previously defined (1), of this concentrate was approximately 200 units/ml.) The 50 ml. of tomatin concentrate was shaken vigorously with 50 ml. of normal butanol, resulting in an almost equal distribution of tomatin activity between the two phases. The butanol layer was removed and placed on a potato starch column prepared as follows: Potato starch (800 g.) was ground with 200 ml. of normal butanol. Butanol saturated with water was added to make a slurry. The potato starch slurry was poured into a glass column (5 cm. diam.) and allowed to settle by gravity. The completed starch column was approximately 60 cm. in length and was washed with butanol saturated with water before the addition of the butanol-tomatine extract. Development of the chromatogram with butanol saturated with water resulted in the separation of some of the coloring matter as a zone near the top of the column as is illustrated in the small column pictured in Fig. 1. Tomatin and rutin were found to occur primarily in the leading zone of the column.

The zones were washed from the column with butanol saturated with water at a flow rate of approximately 12 ml./hr. Samples of the eluate, collected at intervals after color began to appear, were concentrated to dryness and then taken up in hot water. These aqueous solutions were assayed for tomatin activity and the remaining

TABLE I  
*Isolation of Crystalline Rutin from Chromatographic Fractions  
of a Tomatin Concentrate (Potato Starch Column)*

Fraction No.	Butanol-water eluate	Tomatin activity	Rutin crystallized
	<i>ml.</i>	<i>units/ml. eluate</i>	
1	180	6.1	yes
2	10	5.3	yes
3	10	4.9	yes
4	15	4.8	yes
5	10	4.3	yes
6	15	4.1	yes
7	12	3.8	no
8	15	3.4	no
9	160	3.1	no
10	70	1.4	no
11	150	1.5	no
12	50	1.1	no
13	200	1.1	no
14	120	0.8	no

solutions were allowed to stand until rutin crystallized spontaneously in those fractions where it was present in sufficient amount (Table I).

On standing in the refrigerator overnight, rutin crystallized from the water solutions of chromatographic fractions 1 through 6 but only a few crystals appeared in fraction 6. The water solutions of chromatographic fractions 7 through 14 were colored but rutin did not crystallize in any case, even after concentration to a very small volume.

Chromatographic fractions 1 through 6 exhibited identical tomatin activity before and after the crystallization and removal of rutin, indicating that rutin exerts no effect upon the tomatin assay. This conclusion has also been repeatedly confirmed by adding pure rutin to solutions of known tomatin activity.

### *Identification of Rutin*

Approximately 175 mg. of crude crystalline rutin was obtained from chromatographic fractions 1 through 6, the largest amount being obtained from fraction 1. The crude rutin, recrystallized once from hot water, melted at 180–190°C. The ultraviolet spectrophotometric method of Porter *et al.* (6), which involves the measurement of spectral densities of a solution of the anhydrous sample in 95% ethanol, was used for final identification. The spectrum was characterized by absorption maxima near 362.7 and 257.7  $m\mu$ , with specific extinction coefficients 32.3 and 37.5 liter  $g^{-1} cm^{-1}$ , respectively, and an extinction ratio of 0.883 for wave lengths 375.2 and 362.7  $m\mu$ . Highly purified anhydrous rutin has been found by Porter *et al.* (6) to have maxima near 362.7 and 257.7  $m\mu$ , with a specific extinction coefficient of 31.9 at 362.7  $m\mu$  and an extinction ratio of 0.875 at 375.2 and 362.7  $m\mu$ . The results of absorption measurements in the visible range indicated the complete absence of chlorophyll and the presence of some material, soluble in absolute ethanol, which suppresses the curve in the region of 450–660  $m\mu$ .

### DISCUSSION

To determine the amount of rutin present in Red Currant tomato leaves, 500 g. of the same sample used in the chromatographic work reported above was subjected to the procedure developed at the Eastern Regional Research Laboratory for the isolation of rutin. The rutin isolated amounted to 0.037% (moisture-free basis) of the dried Red Currant tomato leaves. This percentage may be considered a minimum value for Red Currant tomato leaves, since it is possible that: (a) enzymatic degradation of rutin may have occurred during the 24-hour period between the harvesting and drying of the plants; (b)

rutin may have been partially destroyed during the drying process; and (c) the tomato plants may not have been harvested at the stage of growth of maximum rutin content. That these factors have an important bearing upon the rutin content of buckwheat has been shown in the detailed studies at the Eastern Regional Research Laboratory (7).

#### ACKNOWLEDGMENT

The authors wish to express their appreciation to Dr. George W. Irving, Jr. for his interest in this work and his helpful suggestions concerning preparation of the manuscript, and to Dr. S. P. Doolittle of the Bureau of Plant Industry, Soils, and Agricultural Engineering for growing the Red Currant tomato plants used in this investigation.

#### SUMMARY

A measurable amount of rutin is present in the leaves of the Red Currant tomato plant (*Lycopersicon pimpinellifolium*). Rutin crystallizes readily from chromatographic fractions having high tomatin activity but rutin does not inhibit the organism (*Fusarium oxysporum* f. *lycopersici*) used for tomatin assay nor does its presence in solution with tomatin appear to influence the assay for tomatin.

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# Chemical Determination of Pleurotin, an Antibacterial Substance from *Pleurotus griseus*

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Received June 13, 1947

## INTRODUCTION

Methods for the chemical determination of the concentration of an antibiotic substance in a culture solution are known for very few. Allinson (1) described a ninhydrin method suitable for crystalline penicillin. The amount of penicillin needed for a determination was between 3 and 9  $\gamma$ . The method, of course, was not specific. The procedure to be described is a simple colorimetric method which is as sensitive as the usual antibacterial method of assay and considerably more accurate.

## EXPERIMENTAL

The principal antibacterial substance<sup>1</sup> produced by the fungus *Pleurotus griseus* grown on a modified Czapek-Dox medium can be obtained in the form of fine yellow needles which contain only the elements carbon, hydrogen and oxygen. The details of production and isolation (3) have been published.

When a 10% aqueous solution of KCN was added to a solution of pleurotin in water or dilute aqueous alcohol, the methylene blue-like color that formed had a broad absorption band between 500 and 750  $m\mu$ . Since the color faded so rapidly that measurement with a photoelectric colorimeter was difficult, a systematic study of the conditions of the development of the color was made. This revealed that both the rate of formation of the color and the rate of fading increased with the concentration of the cyanide. A concentration of 2.5% KCN was optimum for formation of the blue color. At lower concentrations of

<sup>1</sup> Until its chemical structure has been determined, this crystalline substance will be called "pleurotin."



KCN, the amount of color formed from a given amount of pleurotin was reduced. A study of the influence of pH on the reaction between pleurotin and cyanide revealed that the pH should be kept between 10.1 and 10.4. The reaction is incomplete at a lower pH and the destruction of the blue compound increases at higher pH. A reaction time of from 2-4 minutes was satisfactory for tests made at room temperature.

The reaction product can be isolated as a white crystalline solid which shows acidic properties and gives a blue solution at pH greater than 9.

### *Reagents*

*KCN.* 5% aqueous solution of KCN centrifuged to remove suspended matter.

*Blank Buffer.*  $\text{KHCO}_3$  1.5 g.,  $\text{K}_2\text{CO}_3$  3 g.,  $\text{Na}_2\text{HPO}_4$  2.5 g., water 100 ml., pH 10.1.

*KCN—Buffer.* The KCN-reagent is made by mixing equal volumes of the KCN solution and the "blank buffer." The resulting pH is 10.4 as measured by a L. & N. glass electrode.

### *Colorimeter*

A Klett-Summerson photoelectric colorimeter equipped with a No. 66 filter was used in all measurements. The instrument was set to 0 with water in the cylindrical cuvette. The cuvettes were graduated at the 5 and 10 ml. capacities.

### *Standard*

A standard solution containing 2 mg. of pleurotin/ml. of 95% ethanol was prepared and stored at 11°C. in the dark. The working standard was prepared by diluting 5.0 ml. of the concentrated solution to 100 ml. with water and contained 100  $\gamma$  pleurotin/ml. Since solutions of pleurotin decompose when exposed to light, the working standard was prepared freshly each week and was kept at 11°C. in the dark when not in use.

### *Procedure*

The solution containing pleurotin is centrifuged, if necessary, to remove suspended matter. A sample, not more than 1 ml., and usually 0.5 ml., containing less than 0.1 mg. pleurotin is added to each of three cuvettes, 0.5 ml. of the working standard is added to the third cuvette and water is added to each tube to make a total volume of 1 ml.

To the first tube, the blank, is added 1 ml. of the "blank buffer." To each of the other two tubes is added 1 ml. of the KCN-buffer and after standing at room temperature (20-25°C.) for 2 minutes, the solutions are diluted to the 5 ml. mark with distilled water, the contents of the tubes are mixed, and the tubes are read in the colorimeter. The value of the blank is subtracted from each of the other readings.

The calibration curve made by plotting the colorimeter reading, corrected for the blank, against the amount of pleurotin gives a straight line at least to 500  $\gamma$ . In the instrument used, 100  $\gamma$  of pleurotin gave a scale reading of 94.

The third tube, with the added pleurotin, is to test the recovery and to make sure that factors that increase or decrease the color are absent. The increase in the instrument reading caused by the added pleurotin usually is the same as that of the added pleurotin when measured alone.

This chemical method has been used in assaying more than 200 solutions containing from 6–400  $\gamma$ /ml. of pleurotin. It has been a satisfactory and specific method for pleurotin. None of the following antibacterial substances gave a blue color when treated with KCN solution: patulin, penicillin, penicillic acid, kojic acid, gliotoxin, mycophenolic acid, aspergillie acid, citrinin, 2-methyl-1,4-naphthoquinone, *p*-toluquinone, streptothricin, streptomycin, the antibacterial substance from *Arctium minus* and 4 antibacterial substances formed by 3 species of the higher basidiomycetes.

Isolation of crystalline pleurotin in very nearly the amount (85%) to be expected from the results of the cyanide assay of the culture fluid was achieved.

Several culture filtrates were assayed by the cyanide and by antibacterial methods (2) using pure pleurotin as the standard. The antibacterial activity of the solutions could be accounted for by the pleurotin content measured by the chemical method as is shown in the following table.

TABLE I  
*Pleurotin Content of Solutions as Determined by Chemical  
and Bacteriological Methods*  
Pleurotin concentration in  $\gamma$ /ml.

Solution	Assay method	
	Chemical	Bacteriological
69-21	200	50
174	46	100
252	110	100
254	94	100
258	178	100
261	192	100
69-2	260	250
174	92	121
44-8	46	53
Water*	126	125

\* Saturated solution.

*Fluorometric Method*

The reaction product of pleurotin and cyanide shows a bright blue fluorescence when irradiated by the 3660 Å lines of the mercury arc. Measurements in a Klett Fluorimeter using a 597 lamp filter and an 038 photocell filter indicated that the fluorescence method was about 10 times as sensitive as the colorimetric method. The procedure is the same as for the colorimetric method except that the reaction product is diluted to 25 ml. with dilute blank buffer (diluted 10 times with water). The solutions showed concentration quenching of the fluorescence to such an extent that only solutions with a final concentration less than 10  $\gamma$ /25 ml. were suitable for measurement.

## SUMMARY

The antibacterial substance, pleurotin, reacts with alkaline solutions of cyanide to form a blue color which can be used to measure the quantity of it in a solution. The reaction was not given by 17 other antibacterial substances.

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# Effect of Estrogens and Androgens on the Succinoxidase System of Rat Tissues<sup>1</sup>

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Received June 13, 1947

## INTRODUCTION

It is well known that both natural and synthetic sex hormones are important in controlling the function of reproductive tissues. It is conceivable that this control may be effected by the action of the sex hormones on some enzyme system. In a study directed toward finding out whether there is a relation between the action of these sex hormones and the succinoxidase system, McShan and Meyer (1) showed that *in vitro* the synthetic stilbestrol type of estrogen and sodium estrone sulphate inhibit this system in liver and pituitary tissues of the rat. It was of interest, therefore, to extend the *in vitro* study of the determination of the effect of natural and synthetic estrogens and certain androgens on the succinoxidase system of rat tissues, and, in addition, to study the *in vivo* effect of the estrogens on this system. The results are presented in this report.

## EXPERIMENTAL

The tissues were obtained from adult rats of the Sprague-Dawley strain. The animals were killed by decapitation, and samples of the tissues were removed immediately, weighed and placed in 0.1 ml. of cold glass-distilled water contained in a sharp-pointed homogenizing tube. Glass-distilled water was used in making the reagents, the inhibitor solutions, and the homogenates. The tissue was homogenized according to the method of Potter and Elvehjem (2), after which sufficient water was added to make a 5% homogenate, except for kidney and heart tissues where 1% homogenates were used. The amount of homogenate added to the Warburg flasks with the fortified substrate varied, depending upon the tissue, but in the case of liver it was 0.2 ml.

The fortified substrate solution consisted of 1 ml. of 0.1 M phosphate buffer of pH

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<sup>1</sup> Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation, and by the Committee on Research in Endocrinology of the National Research Council.

7.4, 0.3 ml. of 0.5 *M* sodium succinate, 0.3 ml. each of 0.004 *M*  $\text{CaCl}_2$  and  $\text{AlCl}_3$ , and 0.2 ml. of  $3 \times 10^{-4}$  *M* cytochrome *c* (3, 4). This concentration of cytochrome *c* was shown previously (5) to be sufficient to take care of the dilution effect. The proper amounts of 0.001 *M* solutions of the compounds to be tested for inhibitor activity were added to the flasks with the tissue and substrate. Sufficient water was added to make a total volume of 3 ml.; 0.1 ml. of 20% potassium hydroxide was used in the center wells. Determinations were made using duplicate flasks that contained a constant amount of homogenate as it had been shown previously that, under the above conditions, the oxygen uptake is directly proportional to the amount of tissue homogenate used (5).

Approximately 20 minutes elapsed from the time the tissue was removed from the animal until the flasks were placed in the bath of the Warburg apparatus at 38°C. A period of 10 minutes was allowed for the flask contents to come to equilibrium with the temperature of the bath after which the oxygen uptake was determined by taking readings at 10 minute intervals. The results are reported in terms of  $\text{QO}_2$ ; i.e., the mm.<sup>3</sup> of oxygen taken up /mg. of dry tissue/hr. The  $\text{QO}_2$  values are averages based usually on the first four 10 minute periods.

The cytochrome *c* was made from beef heart muscle according to the method of Keilin and Hartree (6), except that the final product was dialyzed against glass-distilled water instead of 1% sodium chloride solution. A 0.5 *M* solution of Eastman sodium succinate was adjusted to pH 7.4 and used as substrate.

A 0.114 *M* solution of Merck ascorbic acid was used as substrate for cytochrome oxidase (7). This experiment was done to determine whether certain of the compounds tested for inhibition acted on cytochrome oxidase. The medium was the same as that for succinic dehydrogenase except 0.3 ml. of  $9 \times 10^{-4}$  *M* cytochrome *c* and 0.2 ml. of 2% liver homogenate were used. To test in another way whether the inhibitor acted on cytochrome oxidase, the cytochrome *c* was replaced by brilliant cresyl blue (8). Five-tenths ml. of a 0.5% solution of the dye was utilized.

The benzenestrol compounds<sup>2</sup> were dissolved in sodium hydroxide to determine whether they had inhibitor properties. Solutions were made by adding 0.3 ml. of water to the proper amount of each compound followed by the addition of 0.03 ml. of 2 *M* sodium hydroxide. The solid particles were dissolved by stirring with a glass rod after which the solution was diluted to 10 ml. to give 0.001 *M* solutions of the compounds. The proper controls with sodium hydroxide were used.

The sodium salts of androsterone sulphate and isodehydroandrosterone sulphate<sup>2</sup> were dissolved in water, as these salts are water-soluble. The phenol and hydroquinone were also dissolved in water, and the thyroxine<sup>3</sup> was dissolved in dilute sodium hydroxide as were the estrogens. A 0.001 *M* stock solution of each of these compounds was made and the proper amount of each solution was added to the Warburg flasks at the time the tests were made.

<sup>2</sup> We are indebted to Dr. A. H. Stuart of the Schieffelin Company for samples of the benzenestrol compounds, to Dr. J. M. Scott of Ayerst, McKenna and Harrison for the two samples of sodium estrone sulphate, to Dr. E. Oppenheimer of the Ciba Company for the sodium sulphate salts of the androgens, to Dr. C. W. Turner, Department of Dairy Husbandry, University of Missouri, for the crystalline thyroxine, and to Dr. C. W. Sondren of the White Laboratories for the stilbestrol type estrogens.

To determine whether the estrogens are inactivated when they inhibit the succinoxidase system the contents of the Warburg flasks were assayed in adult castrate female rats. These castrate rats were first primed with sufficient estrogen to cause about 75% to come into estrus. The total quantity of the estrogen was given in three equal parts at 4-hour intervals. Beginning 18 hours after the last injection vaginal smears were made at 4-hour intervals during the day time for 3 days. One week after the priming injections were started the rats which had responded positively were injected in the same way with the contents of the Warburg flasks after the tissue had reacted for 50 minutes with the estrogen. A mixture of boiled tissue with the fortified substrate plus the estrogen was used as control.

TABLE I

*Inhibition of the Succinoxidase System of Rat Tissues by Diethylstilbestrol*

Kind of rats	Tissue	Dry weight	Homog. used ml.	No. of expts.	QO <sub>2</sub>						
					Control	Molarity (10 <sup>-4</sup> )					
						1.0	2.0				
Adult	Brain	<i>Per cent</i> 21.3	5%	3	40.5	11.8	9.1				
			0.2					5	39.6	27.0	
			0.3								
Adult	Adrenal	30.0	0.2	4	29.7	18.2		1.0			
			0.3	4	30.0						
Young males	Adrenal	30.0	0.2	2	41.2	10.7					
			0.3	3	42.9	31.7					
	Pituitary	21.3	0.2	2	20.0	2.8					
			0.3	2	20.0						
Adult-preg. 12 days	Corpora lutea	22.1	0.2	1	35.9	4.8					
	Ovarian residue	20.6	0.2	1	15.2	0.0					
Adult	Kidney	23.7	1%	2	170.7	15.2			65.9		
			0.2							5	166.5
			0.3							1	185.4
Adult	Heart	23.1	0.2	5	167.2	9.4					
			0.3	7	162.2	14.4					
			0.4	3	153.3	36.6					

To ascertain whether the estrogens inhibit the succinoxidase system *in vivo*, young adult female rats were injected each day for 10 days with 0.25 ml. of oil that contained 25 mg. of diethylstilbestrol. The animals were killed on the 11th day and samples of liver and uterine tissues were assayed for succinic dehydrogenase and cytochrome oxidase. Tissues taken from the same kind of animals that did not receive estrogen were used as controls.

## RESULTS AND DISCUSSION

The results in Table I show that the oxygen uptake was proportional to the amounts of brain, adrenal, pituitary, kidney and heart tissues reacting, as essentially the same  $Q_{O_2}$  values were obtained when different amounts of these tissues were used. The  $Q_{O_2}$  values for the succinic dehydrogenase of adrenal tissue of young male rats are approximately the same as those reported previously (5) and are 25% higher than the value for adrenal tissue of adult rats. The values for pituitary are in agreement with those reported previously (5). The values of the succinic dehydrogenase activity of corpora lutea and

TABLE II  
*Assay of Estrogens Used to Inhibit the Succinoxidase  
System of Rat Liver Tissue*

No. of expts.	Material injected*	Total dose estrogen $\gamma$	No. animals used	Per cent animals in estrus
Diethylstilbestrol				
2	Estrogen	1.5	24	66
4	Boiled tissue plus estrogen	1.5	34	80
3	Flask contents	1.5	28	75
1	Flask contents	15.0	8	100
Sodium estrone sulphate				
1	Estrogen	3.5	23	52
2	Boiled tissue plus estrogen	3.5 and 4	12	42
2	Flask contents	3.5 and 4	12	50

\* The tissue control was prepared by heating the homogenate in a boiling water bath for 10 minutes and mixing with the proper amount of the fortified substrate. The experimental tissue was obtained from the Warburg flasks at the end of runs in which the estrogens were used as inhibitors.

ovarian residue obtained from rats 12 days pregnant are of the same order as the values reported for the 11th day of pregnancy (9).

It appears from the data given in Table I that diethylstilbestrol is not as effective an inhibitor of the succinoxidase system of brain and adrenal tissues as it is of pituitary, lutein, kidney, and heart tissues. The reason for this difference is not apparent.

The question arose as to whether the estrogens are inactivated when they inhibit the succinoxidase system *in vitro*. Experiments were made using diethylstilbestrol and sodium estrone sulphate to ascertain whether inactivation occurred. The results are given in Table II, and they show that these estrogens do not lose estrogenic activity when they are reacted with liver tissue to inhibit the succinoxidase system.

The results recorded in Table III show that diethylstilbestrol in high doses *in vivo* does not cause a decrease in the activity of succinic

TABLE III  
*Effect of Diethylstilbestrol on Succinic Dehydrogenase  
and Cytochrome Oxidase in Vivo*

Exp. no.	Treatment	QO <sub>2</sub> of tissue			
		Liver		Uterine	
		Succ. dehyd.	Cyto. oxid.	Succ. dehyd.	Cyto. oxid.
1	None (control)	94.5	426.8	4.2	78.4
2		72.9	326.8	5.0	76.9
		Av. 83.7	376.8	4.6	77.6
1	Diethylstilbestrol*	72.4	372.4	6.0	83.4
2		65.7	405.4	5.0	63.3
3		74.8	468.6	3.0	71.7
		Av. 71.0	415.5	4.8	72.4

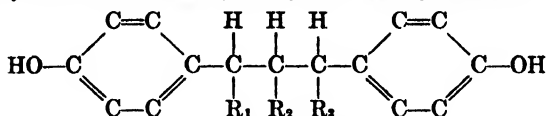
\* Rats were injected with 25 mg. in oil per day for 10 days.

dehydrogenase and cytochrome oxidase of liver tissue as indicated by oxygen uptake *in vitro*. It appears from these results that the estrogens do not exert their estrogenic effect by the *in vivo* inhibition of these two enzymes. It may be that some other enzyme is more sensitive to the estrogens than are the enzymes of the succinoxidase system.



The data shown in Table IV demonstrate that benzeztrol compounds are effective inhibitors of the succinoxidase system of rat liver tissue.

TABLE IV  
*Inhibition of the Succinoxidase System of Rat Liver by Benzeztrol Compounds*



Formula of benzeztrol type compounds

Number compound	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	Estrog. activity mg./RU	No. of runs	QO <sub>2</sub>		
						Control (no inhib.)	Final molarity (10 <sup>-4</sup> )	
							0.5	1.0
020	H	C <sub>2</sub> H <sub>5</sub>	H	5.0	5	73.0	50.6	23.3
103	CH <sub>3</sub>	H	C <sub>3</sub> H <sub>7</sub>	0.2	6	72.4	30.5	3.5
22B	C <sub>2</sub> H <sub>5</sub>	C <sub>2</sub> H <sub>5</sub>	H	0.04	4	73.2	42.4	5.5
32B	C <sub>3</sub> H <sub>7</sub>	C <sub>2</sub> H <sub>5</sub>	H	0.2	5	73.3	20.5	1.1
231B-2	C <sub>2</sub> H <sub>5</sub>	C <sub>3</sub> H <sub>7</sub>	CH <sub>3</sub>	0.005	4	79.4	30.9	2.0
221B-2	C <sub>2</sub> H <sub>5</sub>	C <sub>2</sub> H <sub>5</sub>	CH <sub>3</sub>	0.0008	6	76.0	23.0	1.3

The inhibition by these compounds is of interest, as they are similar in structure to the synthetic stilbestrol estrogens but are not as active in producing vaginal estrus. The formula (Table IV) shows that the benzeztrol compounds have three carbons between the two phenolic rings while the stilbestrol estrogens have only two carbons between the two terminal phenolic groups. In the case of the stilbestrol compounds the groups joined to the two middle carbons are the same as in diethylstilbestrol while the R groups listed in Table IV show that different groups containing from one to three carbons are joined to the three middle carbons of the benzeztrol compounds.

Compound 020 which contains only one ethyl group joined to the middle of the three carbons inhibited to about the same degree as did diethylstilbestrol, hexestrol and dienestrol (1), but this compound has very weak estrogenic properties as compared to these stilbestrols. It is also less active by the vaginal smear test than the other compounds in Table IV which increase in estrogenic activity as the number of alkyl

groups attached to the three middle carbons is increased. These other benztrols are also more effective as inhibitors than compound 020, and they are more effective as inhibitors than the stilbestrols but not as active in producing vaginal estrus. Within this group of estrogens of like chemical structure the inhibition is somewhat correlated with the estrogenic activity of the compounds.

Since it was shown that the inhibitory effect of the stilbestrols on the succinoxidase system was through their action with cytochrome oxidase (1), it was of interest to determine whether the mechanism of inhibition of the benztrol compounds was the same. The data of Table V show that compound 103 inhibited in the presence of cyto-

TABLE V

*Effect of Brilliant Cresyl Blue on the Inhibition of the Succinoxidase System of Liver by a Benztrol Type Compound*

Sod. succ. 0.5 M	Cytochrome c $3 \times 10^{-4}$ M*	Bril. C blue 0.5%	Benztrol No. 103 $1 \times 10^{-3}$ M	QO <sub>2</sub> Average
ml.	ml.	ml.	ml.	
0.3	0.2	—	—	69.4
0.3	0.2	—	0.3	2.8
0.3	—	0.5	—	32.3
0.3	—	0.5	0.3	27.0

\* Final concentration in flasks was  $2 \times 10^{-5}$  M.

chrome c but that it inhibited little in the presence of brilliant cresyl blue. The fact that this dye is autoxidizable in the presence of oxygen permits the succinoxidase system to function without cytochrome oxidase according to Weil-Malherbe (8). Thus, in a system in which cytochrome c is replaced by this dye, if the inhibition is due to the combination of the inhibitor with cytochrome oxidase, the oxygen uptake should be approximately the same both in the presence and absence of the inhibitor. The data in Table V show that this is true for compound 103. This indicates that the benztrols which contain two phenolic groups inhibit the succinoxidase system by reacting with the cytochrome oxidase.

To obtain further proof for the inhibition through cytochrome oxidase, an experiment was made using ascorbic acid as substrate in the presence of cytochrome c (7), both with and without benztrol 103.

The  $Q_{O_2}$  for cytochrome oxidase without the inhibitor was 392 for rat liver as compared with 57 in the presence of the estrogen. These results also show that the inhibition of the succinoxidase system is due to the action of the benzoestrol compounds with cytochrome oxidase, as was found to be the case for the stilbestrols.

In the light of the inhibition caused by the stilbestrols and the benzoestrols, it was of interest to test certain androgens which contain alcoholic instead of phenolic groups. The results given in Table VI show that the sodium sulphate salts of androsterone and isodehydro-

TABLE VI

*Effect of Sodium Sulphate Salts of Androgens and Other Compounds on the Succinoxidase System of Rat Liver*

Compound	No. of expts.	$Q_{O_2}$				
		Control	Final molarity of inhibitor ( $10^{-4}$ )			
			0.5	1.0	2.0	3.0
Androsterone sulphate-sodium salt	3	71.2		70.4	71.6	71.8
Isodehydroandrosterone sulphate-sodium salt	3	71.2		70.0	65.0	73.6
Premarin*	4	67.3		60.6	55.3	
Sodium sulphate salt of estrone	4	74.1		60.2	54.2	
Hydroquinone	6	64.0	5.9	2.5	2.0	
Phenol	4	66.1		69.0	63.7	
			Final molarity of inhibitor ( $10^{-4}$ )			
			1.0	2.0	2.5	3.0
Phenol	2	97.1	96.7	92.3	97.2	94.0

\* An impure sample of sodium estrone sulphate supplied by Ayerst, McKenna and Harrison. The pure sample of sodium estrone sulphate was also supplied by the same company.

androsterone do not inhibit the succinoxidase system when tested in concentrations as high as  $3 \times 10^{-4}$  M. This is a definite difference between these androgens, and the estrogens and hydroquinone which contain two phenolic groups, and sodium estrone sulphate which has only one. The results with sodium estrone sulphate confirm those obtained previously with impure sodium estrone sulphate (1), and they show that it does not inhibit as effectively as the stilbestrols and benzestrols. It is also of interest to note that phenol, which contains one phenolic group, did not inhibit in the concentrations used.

The effect of thyroxine on the succinoxidase system both *in vivo* and *in vitro* was determined since it contains a phenolic group and influences metabolism. The results are given in Table VII. The data show that

TABLE VII  
*Effect of Thyroxine on Succinoxidase System of Rat Liver Tissue*

<i>In vitro</i>						
Treatment	No. of runs	$Q_{O_2}$				
		Control	Thyroxine final molarity ( $10^{-4}$ )			
			0.5	1.0	2.0	3.0
None	6	66.2	—	59.2(4)	59.5(5)	50.8(4)
<i>In vivo</i>						
			Diethylstilbestrol final molarity ( $10^{-4}$ )			
None	2	69.1	42.3	21.9	0.3	
Thyroxine—1 mg. on alternate days for 3 days	2	91.1	60.9	33.9	0.8	

thyroxine moderately inhibits the succinoxidase system *in vitro*. The  $Q_{O_2}$  of liver from uninjected young adult rats is 69.1 as compared to 91.1 for liver obtained from the same kind of animals that had been injected with thyroxine. These results indicate that thyroxine stimulates the succinoxidase system *in vivo*, and are in agreement with the

results reported by Tipton and Nixon (10). Although thyroxine increases the activity of the succinoxidase system when given *in vivo* and at the same time increases the metabolic rate, it inhibits this system *in vitro*, which is probably due to the presence of the phenolic group. Diethylstilbestrol inhibits the succinoxidase of liver obtained both from the treated and untreated rats.

The results obtained with the various compounds used in the inhibition studies indicate that the phenolic group is the primary factor in the inhibition. The inhibition was shown to be through the cytochrome oxidase of the system, and this is not unexpected as this enzyme is known to catalyze the oxidation of certain phenolic compounds, which indicates an affinity of the enzyme for this group. Thus, it appears that the phenolic groups of the estrogenic compounds combine with the active centers and remain attached to the enzyme, which prevents the latter from acting.

Another factor that influences to a certain extent both estrogenic and inhibitory activities of the benzestrol compounds is the number of alkyl groups attached to the three middle carbons. As the number of these groups increases both activities increase but the degree of correlation is better in the case of the estrogenic activity.

The results show that *in vitro* inhibition of the succinoxidase system by diethylstilbestrol and sodium estrone sulphate does not result in inactivation of these estrogens. In this connection it is of interest to point out that Heller (11) inactivated small amounts of estrone by incubation with liver slices, and Zondek, Sulman and Sklow (12), reported the inactivation of estrone and stilbestrol by incubation with rat liver pulp. These workers used large amounts of liver in their inactivation studies while only small amounts of liver tissue are required to demonstrate inhibition of the succinoxidase system by the estrogens. In fact, the small amount of liver tissue used in the Warburg flasks and the short time of incubation may account for the failure of the estrogens to be inactivated even though the conditions were suitable for inhibition of the enzyme by the estrogens. Furthermore, the injection of large amounts of diethylstilbestrol did not cause an *in vivo* decrease in the activity either of succinic dehydrogenase or cytochrome oxidase. These results suggest that the inhibition of the succinoxidase system by various kinds of phenolic estrogens may not be related directly to the basic mechanism by which they produce their estrogenic effect *in*

*vivo*. In the further study of the mechanism by which estrogens produce their endocrine effects the sensitivity of other enzymes to these hormones will be determined.

### SUMMARY

Diethylstilbestrol effectively inhibits the succinoxidase system of rat adrenal, brain, pituitary, lutein, kidney and heart tissues *in vitro*. The system in adrenal and brain tissues appears to be the least sensitive to this compound.

The ability of diethylstilbestrol and sodium estrone sulphate to cause vaginal estrus is not destroyed when they are used *in vitro* to inhibit the succinoxidase system. Furthermore, the injection of large amounts of diethylstilbestrol into rats does not cause a decrease in the activity of succinic dehydrogenase and cytochrome oxidase of liver tissue.

Benzestrol type compounds which contain two phenolic groups and which are less active in producing vaginal estrus than the stilbestrol estrogens were shown to be effective inhibitors of the succinoxidase system of rat liver tissue. The inhibition was shown to be through the cytochrome oxidase of the system.

Hydroquinone was also found to be an effective inhibitor while phenol, which contains only one phenolic group, did not inhibit. Sodium estrone sulphate and thyroxine inhibited, and thyroxine *in vivo* caused the system in liver to become more active.

Androsterone and isodehydroandrosterone which contain alcoholic instead of phenolic groups when tested in the form of the water-soluble sodium sulphate salts did not inhibit.

The results indicate that the phenolic groups of the estrogenic and benzestrol compounds cause inhibition of the succinoxidase system while the alcoholic groups of the androgens do not confer inhibitory properties on these compounds.

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# On the "Pellagrigenic" Inactivity of Indole-3-Acetic Acid in the Rat

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Received June 23, 1947

## INTRODUCTION

In a note, published in October, 1946, Kodicek *et al.* (1) presented data suggesting that the "pellagrigenic" effect of corn in the diet of young rats may be due to its content of indole-3-acetic acid (hetero-auxin, IAA). Krehl *et al.* (2) attempted to replace tryptophan with this substance in the diet of rats deficient in nicotinic acid and tryptophan and found neither stimulation nor further depression of growth. In our own recent studies on the role of pyridoxine in the tryptophan-niacin relationship (3) we administered, along with other tryptophan derivatives, single large doses of indole-3-acetic acid and indole-3-propionic acid and found no increased excretion of nicotinic acid derivatives in the urine (4).

In view of the announcement of Kodicek *et al.* (1) it was decided to determine whether IAA inhibited growth in rats by interfering with the tryptophan-niacin transformation (5). In the experiments described below it was found that IAA administered over a prolonged period to growing rats maintained on a low protein, low niacin diet failed to retard growth, impair food utilization, or produce any discernible reduction in the transformation of tryptophan to niacin.<sup>2</sup>

<sup>1</sup> Nutrition Foundation, Inc., Fellow. Grants in aid of this work are also gratefully acknowledged to the U. S. Public Health Service, The John and Mary R. Markle Foundation, and the Duke University Research Council.

<sup>2</sup> While this paper was in preparation abstracts of papers appeared by W. A. Krehl, A. Carvalho and G. R. Cowgill (*Federation Proc.* **6**, 413 (1947)) and by H. P. Sarett and G. A. Goldsmith (*ibid.*, p. 288), showing results similar to ours.



## EXPERIMENTAL

Male albino rats (Vanderbilt Strain), average weight 60 g., were kept singly in metal metabolism cages with removable tray bottoms. Fresh food and tap water were supplied daily. The animals were weighed every alternate day. The basal diet used in these studies contained casein 10, sucrose 83, salts 4 (6), corn oil 3, L-cystine 0.2 g. supplemented with 1.5 mg. each of riboflavin and pyridoxine, 1.0 mg. thiamine, 200 mg. choline, 6.6 mg. Ca pantothenate, 16 mg. inositol, 15  $\gamma$  biotin, and 133  $\gamma$  vitamin K/100 g. Two drops of cod liver oil were given twice a week. The additions to the basal diet (A) are indicated in Table I. Each rat was given 12 g. of food daily, and on the following day the residue was weighed and discarded. The food consumption in all groups amounted to 10–12 g. per rat per day.

The 48-hour urine specimens were collected under 1 ml. of toluene and 0.5 ml. 1 *N* HCl. The urine and washings were made to a total volume of 50 ml., and after filtration, the samples were stored in the refrigerator.

*N*<sup>1</sup>-methylnicotinamide was determined by the fluorometric acetone-condensation method (7); nicotinic acid by a microbiological procedure employing *L. arabinosus* (8).

In a preliminary experiment in which 1.5 mg.-% indole-3-acetic acid in the basal diet was fed to 10 rats, the growth response equaled that of the control animals. In view of these results, and since Berger and Avery (9) found that corn meal contains 100 mg. indole-3-acetic acid/kg., we thought it desirable to add 10 mg.-% indole-3-

TABLE I  
*Effect of Indole-3-Acetic Acid, Tryptophan and Niacinamide  
on Rats Fed a Low-Protein Diet*

Six rats in each experiment

Diet	Added supplement	Before tryptophan		After tryptophan		Av. excretion of <i>N</i> <sup>1</sup> -methyl-nicotinamide after dose of tryptophan	
		Av. gain g./rat/wk.	Av. diet /g. gain	Av. gain g./rat/wk.	Av. diet /g. gain	Control	50 mg. DL-tryptophan orally
		duration—4 wks.		duration—2 weeks			
A	Basal (10% casein)	13.2	5.9	17	4.5	7/48 hrs. 38 (25–85)	7/48 hrs. 1204 (525–2100)
B	10 mg.-% IAA	12.5	6.3	16	4.8	32 (15–50)	1360 (250–2500)
C*	50 mg.-% L-tryptophan	18.6	4.1			433 (250–550)	
D*	5 mg.-% niacinamide	16.3	4.7			491 (395–620)	

\* The values for groups C and D represent a period of six weeks.

acetic acid to the diet, to provide approximately twice the amount of IAA of the calculated content in a 40% cornmeal diet, assuming a food consumption of 10 g. of diet per rat.

In earlier experiments with low protein diets a number of rats were always found which failed to gain more than 4-6 g. per week. However, if these diets were supplemented with either tryptophan or niacin, all of the animals grew at a normal rate. To examine this effect further, with regard to a possible explanation of the results obtained by Kodicek *et al.* (1), groups C and D, receiving additional tryptophan and niacinamide, respectively, were included in the experiment. The data in Table I show the averaged results for 24 rats under these dietary conditions.

## RESULTS

There was no adverse affect on growth or food consumption in the animals that received indole-3-acetic acid. After 28 days on their respective diets, each rat in groups A and B was given a test dose of 50 mg. DL-tryptophan orally. During the next two weeks these animals were dosed, at suitable intervals, with equal amounts of tryptophan. It can be seen from Table I that the growth response during this period parallels that obtained with groups C and D. Before tryptophan was given, two animals out of six in both groups, A and B, gained only from 3 to 8 g. per week. A prompt increase in the rate of growth was noted in these rats when tryptophan was administered. This might explain why Kodicek and coworkers (11) obtained poor growth in only 60% of their animals maintained on a low protein diet and attributed it to the 1.5 mg.-% indole-3-acetic acid.

To test the effect of IAA on the tryptophan-niacin transformation (5) the excretion of *N*<sup>1</sup>-methylnicotinamide after a 50 mg. dose of DL-tryptophan was determined. The results shown in Table I indicate no significant variation between the IAA and control groups. Nor was there any significant change in the excretion of the total unmethylated nicotinic acid derivatives, as determined by assay with *L. arabinosus*.

The administration of single large doses, 200 mg. of IAA over two days, accompanying the administration of tryptophan did not affect the excretion of nicotinic acid derivatives in the urine.

## DISCUSSION

Although, in the experiments described above, young rats received over 6 times as much IAA (10 mg.-%) as did the rats in the experiments of Kodicek *et al.* (1.5 mg.-%), not only was there no significant effect upon growth and the excretion of nicotinic acid derivatives, but there

were no other toxic manifestations to be observed in appearance, appetite and behavior of the animals. Nor were any toxic effects observed after 200 mg. doses of IAA fed over a period of two days. This occurred in spite of a low protein diet which might be expected to enhance deleterious drug effects. On the basis of these experiments, and in view of the more recent work of Salmon (10), who showed that rats grew satisfactorily on diets containing as much as 92% corn meal and only 4% casein, it is apparent that the pellagra-like syndrome in rats is not due to IAA or another similar substance present in corn. Furthermore, Krehl *et al.* (2) demonstrated amply that the effects of corn meal in rats can be fully reproduced by diets free of corn, but containing other proteins lacking in tryptophan. It would appear as though the ingestion of an amino acid mixture in large amounts increases the demand for tryptophan and/or of nicotinic acid (11). This also appears to be true in the growing chick (12).

#### SUMMARY

The addition of 10 mg.-% indole-3-acetic acid to a low protein diet does not depress the growth rate, nor does it affect the tryptophan-niacin relationship in rats.

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# Electrophoretic Purification of Southern Bean Mosaic Virus<sup>1,2</sup>

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Received July 2, 1947

## INTRODUCTION

In recent studies it was found that preparations of Southern bean mosaic (SBM) virus, isolated from plants grown under conditions of high light intensity, contained a considerable amount of dark brown pigment which could not be removed by precipitation of the virus with  $(\text{NH}_4)_2\text{SO}_4$  or  $\text{MgSO}_4$  (1), by high speed centrifugation (1), nor indeed by crystallization of the virus (2). Preliminary experiments carried out in this laboratory on a highly pigmented preparation of SBM virus in pH 7 phosphate buffer of ionic strength 0.02, showed that the pigmented material migrated faster than the virus in a Tiselius electrophoresis apparatus. This observation suggested that electrophoresis might be used as a tool for further purification of pigmented virus preparations.

## MATERIALS AND METHODS

The Tiselius electrophoresis apparatus (3) used was of the design of Longworth (4, 5) as manufactured by the Klett Manufacturing Company. Standard size, three-piece (4) and four-piece (5) cells were used in the investigations. The temperature was maintained at 2°C. Before electrolysis, each virus preparation was dialyzed for at least 24 hours against a pH 7 potassium phosphate buffer with an ionic strength of 0.02.

Pigment in virus preparations was measured with a Klett photoelectric colorimeter equipped with a red filter. Protein analyses were made by a colorimetric adaptation of the Kjeldahl procedure, involving Nessler's reagent, as described by Miller (6).

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<sup>1</sup> Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

<sup>2</sup> Presented at the 110th meeting of the American Chemical Society, Chicago, Illinois, September, 1946.

<sup>3</sup> Contribution No. 7P47 from the Department of Physics of the University of Pittsburgh.

Virus activity measurements were carried out on bean (*Phaseolus vulgaris* L.) plants. Except when otherwise mentioned, the method described by Price and Spencer (7) was used. Recently (8), it has been shown that this method, when used for measuring (SBM) virus activity, involves an error that seldom exceeds 10 or 15% when the Early Golden Cluster variety of bean is used as a test plant. During the past year, seeds of this variety were unavailable. Consequently, it was necessary to use other varieties that respond to infection by the production of local lesions. In experiments not reported here, it was found that the Kentucky Wonder (green-seeded) variety will serve as a satisfactory substitute for the Early Golden Cluster variety in the measurements of SBM virus activity. It is not unreasonable to assume that the accuracies obtained with the two varieties are comparable.

Virus preparation No. 1 was a highly pigmented preparation, concentrated by chemical precipitation from the sap of diseased plants of the Bountiful variety of beans and brought to the University of Pittsburgh from the Rockefeller Institute laboratories. Portions of it had been crystallized without removal of pigment (2). The concentration procedure involved the following steps: Sap was expressed from diseased bean plants after they had been frozen and subsequently thawed. A second extraction of the pulp was made with a half volume of 5%  $K_2HPO_4$ . The first and second extracts were combined and the flocculent precipitate was removed by centrifugation in an International Clinical Centrifuge equipped with an angle head at 3000 r.p.m. for 15 minutes, and discarded. The virus in the supernatant fluid was precipitated by the addition of 300 g./l. of  $(NH_4)_2SO_4$  and removed by centrifugation at 3000 r.p.m. It was taken up in about one-quarter of the original volume of water and then subjected to an additional precipitation with  $(NH_4)_2SO_4$ . The final preparation was taken up in a volume of water equal to about one-twentieth the volume of the original combined extracts. The concentrated, highly pigmented solution thus obtained was stored in a refrigerator at 3°C. for several months until used in the experiments to be reported.

Virus preparation No. 2 consisted of sap expressed from diseased Bountiful bean plants grown in Pittsburgh and purified in the manner to be described in the next section. Virus preparation No. 3 was obtained in the same manner.

## EXPERIMENTAL RESULTS

### *Concentration of Virus Preparations by Osmotic Means*

An attempt was made to develop a method for the reduction of fluid volume of the virus preparation without resorting to chemical precipitation of the virus or high speed centrifugation. Preliminary experiments involving the method were carried out on virus preparation No. 1. Twenty-four ml. of virus preparation No. 1 were placed in cellophane tubing which was then suspended in about 200 ml. of egg white and held at 4°C. The volume of the virus preparation was estimated at frequent intervals. Results are shown in Table I. It can be seen that, by the end of 9 days, the virus preparation had been reduced twelve-

TABLE I  
*Rate of Fluid Loss During Equilibration of SBM Virus  
Sample No. 1 Against Egg White*

Time in days	Estimated volume of virus preparation ml.
0	24
1	21
2	18
3	12
5	6
6	4
7	3
9	2
13	2

fold in volume. There was, apparently, little or no further reduction in an additional 4 days. This method of reducing volume probably depends upon the differential between the colloidal osmotic pressure of the egg white and that of the virus material. Activity measurements carried out on whole leaves of bean plants indicated no loss of virus during the concentration procedure.

This method of concentrating virus material was utilized in obtaining virus preparation No. 2. About 950 ml. of juice was equilibrated in the same manner as above against one liter of egg white at a temperature of about 4°C. It was observed that the reduction in volume was much less rapid than in the case discussed above. At the end of three weeks, the volume had been reduced to 185 ml., corresponding to about a five-fold concentration. The reduced rate of concentration was probably due to the presence of considerable amounts of low molecular weight protein in freshly expressed juice. Thus, the differential in osmotic pressure between the virus-containing material and the egg white solution was not so great as in the case of virus preparation No. 1, which had been purified to the extent that most of the low molecular weight constituents were no longer present. Virus infectivity tests showed that no virus activity escaped into the egg white during this process. Considerable pigment passed through the dialyzing membrane into the egg white. Aggregated colloidal material and other large-sized particles present in the solution concentrated by dialysis against egg white were removed by centrifugation in a Servall angle centrifuge operated at 5000 r.p.m.

*Electrophoretic Purification of Virus Preparation No. 1*

Virus preparation No. 1, in a phosphate buffer at pH 7 with an ionic strength of 0.02, was electrolyzed for  $6\frac{1}{2}$  hours in a standard size, four-piece electrophoresis cell with a current of .0055 ampere. The pigment was found to move toward the anode at a considerably greater rate than an essentially colorless component, presumed to be the virus itself. The compensator was adjusted to a rate which was just sufficient to maintain the essentially colorless component in a stationary position. At the end of the experiment, the boundaries were arranged in such a manner that the anode arm, consisting of two compartments, was filled with pigment, and the cathode arm was filled with the es-

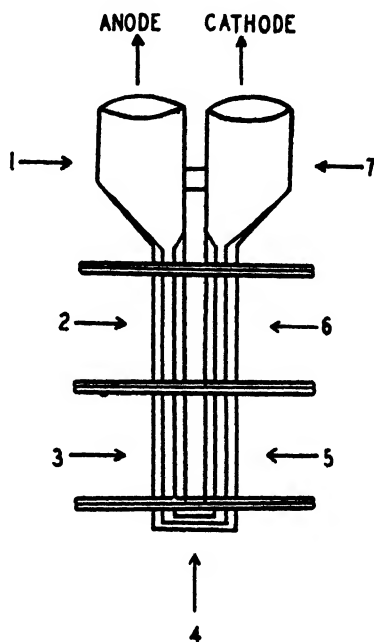


FIG. 1. Longitudinal section of standard four-piece Tiselius electrophoresis cell. Numbers correspond to sampling scheme described in text.

essentially colorless component. The bottom of the cell presumably contained both pigment and colorless component. Seven samples were taken from the cell and tested for pigment, nitrogen concentration, and relative virus concentration.

The 7 samples were numbered as follows: 1, Contents of adapter connected to anode electrode vessel; 2, Upper half of electrophoresis cell on anode side; 3, Lower half of electrophoresis cell on anode side; 4, Bottom of electrophoresis cell; 5, Lower half of cell on cathode side; 6, Upper half of electrophoresis cell on cathode side; and 7, Contents of adapter connected to cathode electrode vessel. This system of numbering is illustrated diagrammatically in Fig. 1. The original virus material prior to electrolysis was designated sample 8. The results of the analyses are shown in Table II. It can be observed, first of all, that the pigment,

TABLE II  
*Electrophoretic Fractionation of SBM Virus Preparation No. 1*

Sample no.	Pigment (colorimeter reading)	Nitrogen (mg./ml.)	Virus activity (Per cent of original concentration)
1	66	.066	0
2	139	.154	0
3	142	.285	16
4	283	.44	110
5	52	.59	101
6	33	.71	83
7	0	.005	6
8	374	1.08	100

after electrolysis, was contained largely in the anode electrode vessel, in the upper and lower halves on the anode side, and in the bottom of the electrophoresis cell. On the other hand, the bulk of the virus was contained in the cathode side and in the bottom of the electrophoresis cell. Furthermore, from the analyses on samples 1 and 2, a pigment concentration corresponding to a colorimeter reading of 100 has associated with it about 0.105 mg./ml. of nitrogen. Since the colorimeter reading on the original virus preparation was 374 units,  $374/100 \times 0.105$  or 0.39 mg./ml. of nitrogen in the original virus was associated with pigmented material. Nitrogen analyses on the original material showed that it contained 1.08 mg. of nitrogen/ml. Thus, about 36% of the nitrogen in the original preparation was associated with material which moved with the pigment during electrolysis. These calculations are based on the assumption that the ratio of pigment to nitrogen is a constant for the pigmented material.



If the analytical results on samples 5 and 6 are averaged, one can see that the pigment concentration was reduced to about 11% of the original, the nitrogen concentration was reduced to about 60% of the original, but the virus infectivity was not reduced substantially. If 11% of the original pigment remained in the composite of samples 5 and 6, and if 0.39 mg./ml. of the original nitrogen was associated with the pigmented material, 0.04 mg./ml. of the residual nitrogen in the composite of samples 5 and 6 was still associated with extraneous pigmented material. Since the average nitrogen concentration of samples 5 and 6 was 0.56 mg./ml. on a nitrogen basis, the purified virus was not more than  $\frac{(0.65 - 0.04) \times 100}{0.65}$  or 94% pure. Four additional

similar electrophoresis experiments were carried out on virus sample No. 1. Qualitatively similar results were obtained. In all cases, there was no significant decrease in virus concentration in the cathode half of the electrophoresis cell. The pigment reduction varied from 75% in the first exploratory experiment to 96% in the most favorable experiment. On the basis of the reasoning outlined above, this best preparation would have had an estimated purity of about 98%.

The results obtained in the 5 experiments carried out with virus preparation No. 1 show conclusively that, by means of electrolysis of highly pigmented SBM virus preparations, it is possible to obtain fractions which have lost up to 96% of their pigment, but which have suffered only small decreases in virus activity. The essentially colorless fractions obtained in two experiments were pooled and were then subjected to electrophoresis experiments, this time, with the object in view of determining the homogeneity and the mobility of the material. Schlieren scanning diagrams of the boundary were obtained by the Longworth-Schlieren scanning method. Tracings of the original photograph are shown in Fig. 2. It is evident that the material is essentially

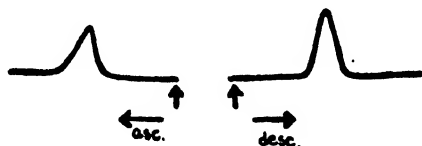


FIG. 2. Tracings of Schlieren scanning diagrams of ascending and descending boundaries in electrophoresis experiment on Southern bean mosaic virus. Vertical arrows indicate starting positions. Scale 1 = 1.

homogeneous with respect to electrophoretic mobility. The average value of the mobility in both forward and reverse directions was found to be  $5.08 \times 10^{-8}$  cm./sec./volt/cm. This value is in good agreement with that obtained by Miller and Price (9) on a preparation of SBM virus isolated by centrifugal means. In view of the fact that this essentially pigment-free preparation contains the bulk of the virus activity in the original virus preparation, and of the fact that it is an electrochemically homogeneous material with an electrophoretic mobility comparable to that of SBM virus preparations described by Miller and Price, it seems reasonable to assume that the essentially pigment-free material represents a virus preparation of a reasonable degree of purity. The degree of resolution obtained probably would not be able to indicate impurities present to the extent of much less than 5%.

*Electrophoretic Purification of Southern Bean  
Mosaic Virus Preparation No. 2*

Virus preparation No. 2, which was concentrated by means of equilibration against egg albumin and subsequent centrifugation at 5000 r.p.m. was dialyzed for 3 days against potassium phosphate buffer at pH 7 and ionic strength 0.02. This material was then electrolyzed in a standard four-piece electrophoresis cell for  $6\frac{1}{4}$  hours with a current of 0.0073 ampere. At the end of the experiment, the cathode arm of the electrophoresis cell, consisting of two compartments, was relatively free of pigment. Two Schlieren boundaries were observed. They were moved with the compensator until one was just beneath the top of the upper cathode half of the cell. The anode arm and the adapter connecting with the anode electrode vessel contained considerable amounts of pigment. Seven samples were withdrawn, as described for the experiment on virus preparation No. 1. Nitrogen analyses and virus activity measurements were made on all 7 and compared with the original material, designated as sample 8. Pigment analyses were made on 3 of the samples. The results are shown in Table III. They can be interpreted to indicate that virus preparation No. 2 consisted of at least 3 components; an essentially colorless component which migrated toward the anode at a higher rate and which seemed to be of protein nature, but which did not seem to possess appreciable virus activity, a more slowly migrating essentially pigment-free material, and pigmented material. The more slowly moving essentially pigment-free

TABLE III  
*Electrophoretic Fractionation of Virus Preparation No. 2*

Sample no.	Pigment (colorimeter reading)	Nitrogen (mg./ml.)	Virus activity (Per cent of original)
1		.132	0
2		.64	0
3		.82	23
4		.93	177
5	84	1.05	88
6	15	.72	116
7		.05	0
8	305	1.40	100

material possessed about half of the original nitrogen, essentially full virus activity, and only about 5% of the original pigment concentration. Therefore, this fraction can be presumed to be the one that contained the virus in the most highly purified state.

To determine whether or not the purified preparation represented reasonably pure virus, it was mixed with the essentially colorless preparation obtained by electrolysis of preparation No. 1. This material, it will be remembered, was shown to be electrochemically homogeneous and to possess a mobility comparable to that previously found for SBM virus. A portion of the composite virus, corresponding to 2.37 mg. of nitrogen, was mixed with a portion of the purified material of preparation No. 2, corresponding to 1.77 mg. of nitrogen, and made up to 11 ml. This material was equilibrated with pH 7 phosphate buffer of ionic strength 0.02. It was electrolyzed at 2°C. for 17½ hours with a current of 0.0018 ampere. A single boundary was maintained throughout the electrolysis. The mobility was in reasonable agreement with the value previously reported by Miller and Price (9). This experiment demonstrated that the bulk of the material in this purified preparation was electrochemically indistinguishable from the purified material obtained from preparation No. 1, and that it could therefore be presumed to be a reasonably well purified preparation of SBM virus.

As can be seen in Table III, fraction 6 of virus preparation No. 2 contained 0.72 mg. of nitrogen/ml. Since this has been shown to be probably fairly well purified virus, it can be computed from the nitrogen analyses of Miller and Price (10) that this corresponds to  $100/17 \times 0.72$  or 4.2 mg. of virus/ml. Since the original plant juice was concentrated

5-fold, this would correspond to a yield of 0.84 mg./ml. of original plant juice. This is about twice the maximum yield obtained in the original investigation (1).

A second electrophoresis experiment was carried out on virus preparation No. 2 with substantially the same results. Similarly, an electrophoretic purification study of preparation No. 3 yielded comparable data.

#### DISCUSSION

Experiments reported in this communication have demonstrated that the electrophoresis apparatus can be used to further purify virus materials in some cases in which standard physical and chemical procedures fail. The most conspicuous limitation of the electrophoresis method, as carried out with readily available standard equipment, is that only small volumes of material can be utilized. It is possible to obtain about 7 ml. of purified material in a single experiment. This limitation is offset in part by the relatively high fraction of the virus which can be recovered in the purified material. Almost all fractionation procedures, whether chemical or physical, involve considerable losses of the desired constituent. Exceptions are those cases in which extreme differences in properties exist between the desired constituent and the undesired components. In the electrophoretic method, the loss of virus has been demonstrated to be of the order of 10%. The small volume of final material is not necessarily serious, because, by the method of osmotic concentration, it is possible to reduce the volume of a preparation to such an extent that a very considerable amount of virus can be obtained in 7 ml. It would be easy to devise experiments in which between 100 and 200 mg. of material could be purified in this manner. When one is dealing with animal viruses, it is unusual to have even this amount available.

#### ACKNOWLEDGMENT

The authors wish to acknowledge the technical assistance of Miss Harriet L. Carnelly and Miss Betty Holt.

#### SUMMARY

A preparation of Southern bean mosaic virus, which contained pigment not removable by centrifugal fractionation or even by crystallization, was purified by means of electrolysis in the standard Tiselius

electrophoresis apparatus. It was found possible to concentrate solutions of Southern bean mosaic virus by merely equilibrating across a cellophane membrane with the white of eggs. After concentrating in the manner indicated above, two preparations of purified Southern bean mosaic virus were obtained by subjecting juice from infected bean plants to electrolysis in the Tiselius electrophoresis apparatus.

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# Isotopic Composition of Acetylmethylcarbinol Formed by Yeast Juice

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Received June 12, 1947

## INTRODUCTION

No general agreement exists as to the mechanism of the biological formation of acetylmethylcarbinol. Considerable evidence has been presented (8, 9) to support the view that acetaldehyde is an intermediate in the formation of the carbinol from glucose. Others have reported the formation of acetylmethylcarbinol from pyruvic acid (1). If a three-carbon compound, such as pyruvate, combines with labeled acetaldehyde ( $C^{13}H_3C^{13}HO$ ), to form acetylmethylcarbinol and  $CO_2$ , the carbinol should contain heavy carbon in only one end of the molecule.

The purpose of this paper is to report on the isotopic composition of the acetylmethylcarbinol formed by yeast juice from  $C^{13}$  acetaldehyde in the presence of pyruvic acid (6).

## EXPERIMENTAL

Heavy carbon acetylmethylcarbinol was prepared by an active dried yeast juice from heavy carbon acetaldehyde and pyruvate (6). The heavy carbon acetaldehyde served as a tracer.

The acetylmethylcarbinol formed by the yeast juice was purified before degradation. The fermentation liquor (120 ml.) was made acid to congo red and distilled to 20 ml., then steam-distilled and 200 ml. collected. Removal of the acetylmethylcarbinol by alkaline distillation followed by steam distillation was not satisfactory because of the large amount of ammonia passing over. To remove the volatile acids that were subject to being carried over, the distillate (200 ml.) was made alkaline to phenolphthalein and distilled to 20 ml., and then steam-distilled until a total of 400 ml. of distillate were collected. This "alkaline distillate" was refluxed for 2 hours to remove certain sulfur compounds present in the steam.

### *Degradation Procedure*

The methyl group adjacent to the keto carbon was split off by the iodoform reaction according to the method of Langlykke and Peterson (7). An aliquot of the "alkaline

distillate" was made alkaline with  $\text{CO}_2$ -free  $\text{NaOH}$  and the iodine solution was added drop by drop with constant agitation. The reaction was allowed to continue for 15 minutes when just enough acid ( $\text{H}_2\text{SO}_4$ ) was added to neutralize the sodium hydroxide. Then 0.1 ml. 6  $N$   $\text{H}_2\text{SO}_4$  excess was added to acidify the mixture. The excess iodine was titrated with sodium thiosulfate. No starch was added as an indicator, and therefore, the end-point was not sharp. The iodoform was filtered through a sintered glass crucible, washed and dried for two days in a desiccator over  $\text{CaCl}_2$ . After weighing, the dried iodoform was treated with 6  $N$   $\text{H}_2\text{SO}_4$  and oxidized by the Friedemann and Kendall (3) method. The  $\text{CO}_2$  was collected in  $\text{CO}_2$ -free  $\text{NaOH}$ . In later experiments the iodoform was collected on asbestos pads and, when dry, oxidized directly, omitting the  $\text{H}_2\text{SO}_4$  step, since considerable iodoform escapes during this process.

The filtrate from the iodoform filtration, which contains the lactic acid, was treated with  $\text{AgNO}_3$  (10%) to remove the  $\text{HI}$ . The precipitate was filtered out and the  $\text{Ag}$  was removed with  $\text{HCl}$  and the precipitate again removed by filtration. Experiments showed that iodine interfered with the oxidation of the lactic acid and thus had to be removed.

The lactic acid was oxidized by a modification of the Friedemann and Graeser (4) method. The solution containing the lactic acid was placed in a 300 ml. Erlenmeyer flask and 10 ml.  $\text{MnSO}_4$  were added. The flask was connected to a water-cooled reflux condenser attached to a bead tower containing sodium bisulfite.  $\text{CO}_2$ -free air was slowly passed through while  $\text{KMnO}_4$  was added dropwise until a brown color appeared. The  $\text{CO}_2$  was collected in a bulb containing  $\text{CO}_2$ -free  $\text{NaOH}$ . Between the sodium bisulfite tower and the  $\text{NaOH}$  tower was a bead tower containing saturated  $\text{KMnO}_4$  to collect  $\text{SO}_2$  from the bisulfite.

In later experiments the lactic acid solution was reduced in volume by acidifying to congo red and evaporating to 25 ml. This solution was steam distilled and 250 ml. of distillate collected. The residue was made alkaline and was ether-extracted continuously for 12 hours. The ether fraction was acidified and again extracted for 11 hours. The ether was distilled off and the solution evaporated to half volume.  $\text{AgNO}_3$  was added and the precipitate filtered. The  $\text{Ag}$  was removed with  $\text{HCl}$ .  $\text{MnSO}_4$  was added and the mixture refluxed with  $\text{CO}_2$ -free air for 15 minutes.  $\text{KMnO}_4$  was then added and the procedure followed as before.

The acetaldehyde obtained from the oxidation of the lactic acid was degraded by the iodoform reaction.

The formic acid in the filtrate from the iodoform reaction was made acid to congo red and distilled to 25 ml., and then 500 ml. of distillate collected by steam distillation. This distillate was refluxed for 2 hours.

The distillate was neutralized to phenolphthalein, a slight excess of  $\text{NaOH}$  was added, and the solution boiled down to 200 ml. The solution was distilled to 15 ml., cooled, and acidified with  $\text{H}_2\text{SO}_4$  to congo red. The acidified solution was steam distilled and 200 ml. distillate collected, which was refluxed for 15 min. and cooled. Two g. of  $\text{HgO}$  were added and the flask attached to the  $\text{CO}_2$  outfit. The mixture was refluxed for 20 minutes with  $\text{CO}_2$ -free air passing through. Five ml. of 25% phosphoric acid were added and the aeration and boiling continued for another 15 min. The  $\text{CO}_2$  formed was collected in  $\text{CO}_2$ -free  $\text{NaOH}$ .

In other experiments the acetylmethylcarbinol was cleaved at the center of the molecule by the periodate oxidation to yield acetaldehyde and acetic acid. An aliquot of the purified acetylmethylcarbinol was oxidized with  $\text{KIO}_4$  according to the method of Stahly and Werkman (10).

The residue obtained from the  $\text{KIO}_4$  oxidation was made acid to congo red, distilled to 15 ml. and distilled for 2 hours. The solution was made alkaline to phenolphthalein and evaporated to 10 ml. This was made acid to congo red and steam distilled to 100 ml. and the distillate refluxed for 15 minutes, neutralized and evaporated to dryness. The residue was taken up in  $\text{CO}_2$ -free  $\text{H}_2\text{O}$  and filtered. This solution was then oxidized by persulfate to  $\text{CO}_2$ .

In all cases the  $\text{CO}_2$  from the oxidation procedures was liberated from the sodium bicarbonate with 4 *N* lactic acid into a previously evacuated extraction bulb containing anhydrous. The  $\text{CO}_2$  was then analyzed on the mass spectrometer and the  $\text{C}^{13}$  determined.

TABLE I

*Degradation of Heavy Carbon Acetylmethylcarbinol Formed from Heavy Carbon Acetaldehyde ( $\text{C}^{13}\text{H}_5\text{C}^{13}\text{HO}$ ) by Yeast Juice*

Compound	Part of A.M.C.	Millimoles	Excess $\text{C}^{13}$
Acetylmethylcarbinol used	$\text{CH}_3\text{COCHOHCH}_3$	0.17	0.88
Iodoform	$\text{CH}_3$ of $\text{CH}_3-\overset{\text{O}}{\underset{\text{  }}{\text{C}}}-$	0.11	0.10
Lactic acid formed	$\begin{array}{c} \text{O} \quad \text{OH} \\ \text{  } \quad   \\ -\text{C}-\text{C}-\text{CH}_3 \\   \\ \text{H} \end{array}$	0.12	—
$\text{CO}_2$ from lactic acid	$\begin{array}{c} \text{O} \\ \text{  } \\ -\text{C}- \end{array}$	—	0.26
Iodoform from acetaldehyde from lactic acid	$\text{CH}_3$ of $\text{CH}_3-\overset{\text{OH}}{\underset{ }{\text{C}}}-$	0.10	0.27
Formic acid from acetaldehyde from lactic acid	$\begin{array}{c} \text{OH} \quad \text{OH} \\   \quad   \\ -\text{C}- \text{ of } \text{CH}_3-\text{C}- \\   \quad   \end{array}$	—	0.74
Acetaldehyde from $\text{KIO}_4$ oxidation of A.M.C.	$\text{CH}_3-\overset{\text{OH}}{\underset{ }{\text{C}}}-$	0.02	0.34



*Degradation of Heavy Carbon Acetylmethylcarbinol*

In Table I, 0.17 mM of acetylmethylcarbinol was used, containing an excess  $C^{13}$  content of 0.88%. The iodoform, originating in the acetyl group, was 0.11 mM and contained 0.10 atom-% excess  $C^{13}$ . Experiments with ordinary acetylmethylcarbinol always gave low yields of iodoform. This compound is so volatile that the deficiency could well be explained by evaporation during the drying procedure; however, only 0.12 mM of lactic acid was formed. The iodoform arising from the acetaldehyde obtained from the lactic acid contains the carbon of the methyl group adjacent to the carbinol carbon.

There was 0.10 mM of iodoform recovered containing 0.27% excess heavy carbon. The amount of  $CO_2$  from the lactic acid was not determined, but the excess  $C^{13}$  content was 0.26%. The formic acid obtained from the degradation of the acetaldehyde from the lactic acid contained 0.74% excess heavy carbon.

The original acetylmethylcarbinol contained 0.88% excess heavy carbon and the methyl group next to the carbonyl carbon contained 0.10% excess heavy carbon. The  $CO_2$  (carbonyl carbon) contained 0.26. The formic acid (carbinol carbon) contained 0.74% excess heavy carbon which is closest to the per cent excess heavy carbon in the original acetylmethylcarbinol molecule.

The heavy carbon from the heavy carbon aldehyde has gone into all carbons in the acetylmethylcarbinol. If one assumes the acetylmethylcarbinol is formed from one biologically active acetaldehyde molecule and an added acetaldehyde molecule, it is to be expected that one end of the acetylmethylcarbinol will contain the increased concentration of heavy carbon, provided, of course, that acetylmethylcarbinol was not reduced to 2,3-butylene glycol (or oxidized to diacetyl) and oxidized (reduced) again to acetylmethylcarbinol. Thus, if acetylmethylcarbinol were oxidized with KIO and the molecule split to acetaldehyde and acetic acid, one compound would contain the increased heavy carbon. Eight ml. of the fermentation liquor, containing 0.02 mM of acetylmethylcarbinol, were oxidized to  $CO_2$  by persulfate and the amount of heavy carbon determined. The excess heavy carbon contained in the acetaldehyde, which arose from the carbinol end of the acetylmethylcarbinol molecule, was 0.34. This was only slightly higher than that obtained in the previous degradation procedures for the two carbons.

The decrease in heavy carbon content may be attributed to CO<sub>2</sub> dilution caused by procedures used in degrading the compounds. It will be observed in Table I that a relatively small amount of acetylmethylcarbinol was used. All experimental procedures were rechecked to determine whether there were any dilution effects during degradation of ordinary acetylmethylcarbinol. Only a slight dilution effect could be detected.

An experiment was designed to minimize the small CO<sub>2</sub> dilution effect and determine the C<sup>13</sup> content of each half of the acetylmethylcarbinol molecule. The remaining alkaline filtrates, containing the heavy carbon acetylmethylcarbinol, were oxidized by periodate. Sixty-five hundredths of a millimole of C<sup>13</sup> acetylmethylcarbinol were used.

TABLE II

*Degradation of Acetylmethylcarbinol by Periodate Oxidation*

Compound	Part of A.M.C.	Millimoles	Excess C <sup>13</sup>
A.M.C.	whole	.65	1.05
Acetaldehyde	$\begin{array}{c} \text{H} \\   \\ \text{CH}_3-\text{C}-\text{OH} \\   \end{array}$	.62	0.86
Acetic Acid	$\begin{array}{c} \text{CH}_3-\text{C}=\text{O} \\   \end{array}$	—	0.94

The amounts of C<sup>13</sup> in the compounds are recorded in Table II. The whole acetylmethylcarbinol molecule contained 1.05 atom-% excess C<sup>13</sup>. The acetaldehyde, representing the carbinol end of the molecule, contained 0.86% excess C<sup>13</sup> and the acetic acid, representing the carbonyl end of the molecule, contained 0.94% excess C<sup>13</sup>.

These data indicate the C<sup>13</sup> is evenly distributed among the carbon atoms of the molecule.

A larger quantity of heavy carbon acetylmethylcarbinol was degraded using the same procedures. The data (Table III) indicate that 0.53 mM of acetylmethylcarbinol were employed; 0.45 mM of iodoform were obtained from the addition of iodine to the alkaline distillate of the carbinol. There was 0.46 mM of acetaldehyde and 0.60 mM of CO<sub>2</sub> from the oxidation of the lactic acid formed by the iodoform reaction. An aliquot of aldehyde was oxidized directly from the bisul-

TABLE III  
*Degradation of Heavy Carbon Acetylmethylcarbinol*

Compound	Part of the A.M.C. Molecules	Millimoles	Excess C <sup>13</sup>
Acetylmethylcarbinol	whole	0.53	1.44
Iodoform	CH <sub>3</sub> of CH <sub>3</sub> -C(=O) 	0.45	1.26
Aldehyde from lactic acid	H   CH <sub>3</sub> -C-OH 	0.46	2.02
CO <sub>2</sub> from lactic acid	O    -C-	0.60	0.54
Aldehyde from lactic acid (liberated from bisulfite before persulfate oxidation)	H   CH <sub>3</sub> -C-OH 	—	2.07

fite by persulfate oxidation. Another aliquot of aldehyde was liberated from the bisulfite before oxidation.

The percentage excess C<sup>13</sup> content of the acetylmethylcarbinol molecule was 1.44; the iodoform 1.26 and the CO<sub>2</sub> from the lactic acid was 0.53. The aldehyde from the lactic acid was 2.02% excess C<sup>13</sup> for the sample oxidized directly from the bisulfite and 2.07% for the sample liberated from the bisulfite before oxidation. These data indicate that the heavy carbon contents of the various carbon atoms differ. The carbonyl end of the molecule contains the smaller quantity of heavy carbon. The carbinol end contains a greater percentage than the whole molecule, indicating a greater fixation of the added acetaldehyde in the carbinol end of the molecule.

#### DISCUSSION AND SUMMARY

The Dirscherl (2) scheme for the synthesis of acetylmethylcarbinol requires the occurrence of C<sup>13</sup> in only one end of the carbinol molecule when C<sup>13</sup>H<sub>5</sub>C<sup>13</sup>HO is used as the tracer. If the acetylmethylcarbinol is reduced or oxidized to a symmetrical molecule, the distribution of the heavy carbon will occur among all four carbon atoms. The data are in agreement with this concept, and it appears likely that acetylmethyl-

carbinol is formed by the condensation of pyruvic acid and acetaldehyde. The heavy carbon-enriched acetylmethylcarbinol formed by the yeast juice was degraded and an increased concentration of heavy carbon was formed in all four carbon atoms. The distribution was not, however, equally divided among the four carbon atoms and was such as would be expected from a partial conversion of acetylmethylcarbinol to a symmetrical molecule.

On the other hand, the data do not exclude the existence of other methods of formation of acetylmethylcarbinol. If there were random selection of the acetaldehyde in the condensation of two acetaldehyde molecules to form the carbinol, then the heavy carbon atoms would be equally distributed. This was not the case.

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# **The Interrelations of Choline and Methionine in Growth and the Action of Betaine in Replacing Them**

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Received May 20, 1946

## **INTRODUCTION**

This report deals primarily with the interrelations of choline and methionine in the growth of normal chicks during the fourth week of life and, to a lesser extent, with the reactions with them of serine, glycocyamine, homocystine and glycine betaine. The regulation of food consumption is briefly discussed. Choline and methionine have at least two roles in metabolism: those of tissue elements and of methyl donors. Whether these roles are mutually exclusive is uncertain, but there is some evidence that they are (1). It is not known whether both the compounds transfer methyl groups to their ultimate acceptors or whether one methylates another. It has been shown, however, that the methylation of glycocyamine to creatine by liver slices is accelerated by methionine, but not by choline or betaine (2). Evidence of some of these and other points will be furnished.

## **EXPERIMENTAL PROCEDURE**

The birds used were single-comb White Leghorns. They were fed a commercial-type rearing diet for the first three weeks of life (the pretest period) and the test diet during the fourth week. The method of selecting the birds and measuring growth has already been described (3, 4).

### *Diets*

*The practical rearing diet* contained (per cent) ground grains (50), wheat bran (15), heated soybean meal (10.5), alfalfa meal (7.5), fishmeal (5.5), dried whey (5), bone-meal, oyster shell and salt (3.2), fortified sardine oil containing 1000 units of vitamin A and 400 units of vitamin D/g. (0.25).

*The basal diet during the test period* (described in Ref. (4)) contained isolated soybean protein equivalent to 0.30% methionine, 0.05% cystine and negligible choline.

In a few cases arachin, prepared from peanut meal (5), was substituted for soybean protein and was equivalent to 0.15% methionine and 0.20% cystine. The arachin-containing basal diets were supplemented with L-tryptophan 0.2%, L-lysine 0.5%,

TABLE I  
*Dietary Supplements and Chick Weights and Growth*

1	2	3	4	5	6	7	8
Hatch and Pen No.				Initial Weight	Growth		Av. Food Consumed/ Bird/Day
	L-Cystine	Choline-Cl	DL-Methionine		Experimental	Adjusted to In. Wt. 100 g.	
	Concentration in diet, per cent			g.	per cent	per cent	g.
			<i>Section A</i>				
III 12	0.70	0.015	0.14	100.8	-2.17	-2.18	—
III 13	0.55	0.015	0.40	119.0	3.48	3.81	16.3
III 14	0.55	0.015	0.70	109.6	4.54	4.71	16.0
VII 11	0.55	0.015	1.28	135.0	4.68	5.29	19.0
III 6	0.70	0.10	0.14	102.5	-1.31	-1.27	6.2
III 7	0.55	0.10	0.40	117.9	4.88	5.19	18.0
VII 10	0.55	0.10	0.57	142.8	4.98	5.73	23.0
III 8	0.55	0.10	0.70	112.1	6.00	6.21	18.5
VII 9	0.55	0.10	1.00	135.6	4.80	5.42	20.2
VII 5	0.55	0.20	0.30	143.2	3.79	4.55	18.7
IV 1	0.55	0.20	0.50	137.6	4.89	5.55	19.3
VII 4	0.55	0.20	1.00	145.6	4.44	5.24	20.8
IV 2	0.55	0.20	1.28	125.8	4.00	4.45	—
V 2	0.55	0.20	1.50	131.3	3.50	4.05	15.2
VII 2	0.55	0.32	0.62	140.3	5.36	6.07	20.3
III 1	0.55	0.40	0.14	103.7	-0.70	-0.64	7.3
V 1	0.55	0.40	0.70	138.1	4.98	5.65	19.3
VII 6	0.55	0.47	0.40	138.1	4.89	5.56	20.3
V 7	0.55	0.50	0.30	126.4	4.27	4.73	—
VII 7	0.55	0.50	1.28	128.4	3.45	3.95	19.5
VII 8	0.55	0.64	0.61	142.1	4.89	5.63	21.0
VII 1	0.55	0.69	1.00	135.7	3.77	4.40	18.3
VIII 1	0.55	0.94	0.30	125.1	4.27	4.71	—
IV 3	0.55	0.94	0.50	135.8	5.55	6.18	20.3
VIII 3	0.55	0.94	0.70	130.2	4.65	5.18	—
IV 5	0.55	0.94	1.28	130.8	2.81	3.35	12.7
V 8	0.55	1.20	0.40	139.9	4.86	5.56	19.5
VII 3	0.55	1.60	0.40	141.2	4.88	5.62	22.3

TABLE I—(Continued)

1	2	3	4	5	6	7	8
Hatch and Pen No.				Initial Weight	Growth		Av. Food Consumed/ Bird/Day
	L-Cystine	Choline-Cl	DL-Methionine		Experimental	Adjusted to In. Wt. 100 g.	
	Concentration in diet, per cent			g.	per cent	per cent	g.
			Section B				
V 5*	0.55	0.20	1.50	124.0	4.54	4.96	15.2
VII 12*	0.55	0.20	1.50	125.4	4.02	4.46	15.5
V 4*	0.55	0.20	1.50	129.1	4.75	5.26	16.3
VII 13*	0.55	0.20	1.50	126.9	4.40	4.87	17.3
VII 14*	0.55	0.20	0.70	132.5	4.70	5.27	20.3
VII 15*	0.55	1.30	0.70	141.4	3.25	3.98	18.5
V 3*	0.05	0.20	1.50	129.6	3.11	3.63	14.7
V 6	0.05	0.20	1.50	122.1	3.94	4.33	14.7
			Section C				
III 11*	0.55	0.015	0.70	118.7	4.57	4.90	14.7
III 2*	0.55	0.10	0.40	110.1	5.31	5.50	17.2
III 3*	0.55	0.10	0.70	112.4	4.86	5.08	16.5
III 5*	0.55	0.10	0.30	107.0	4.63	4.75	13.7
III 4*	0.55	0.10	0.30	115.6	4.72	4.99	17.7
IV 4*	0.55	0.20	0.50	135.1	5.01	5.62	—
VII 16*	0.55	0.10	0.70	137.0	3.76	4.41	18.2
			Section D				
IV 8}	Diet: U. C. Chick Mash			130.5	6.29	6.82	—
V 10}				135.0	6.13	6.74	—
VII 36}				138.0	6.12	6.79	23.0

Hatching dates: III: 1-10-45; IV: 2-7-45; V: 3-7-45; VII: 4-18-45; VIII: 5-12-45.

\* Additional supplements: Serine: V-5 and V-6: 0.52; VII-12: 1.03 Glycocyamine: V-4: 0.58; VII-13: 1.15. DL-Homocystine: VII-14 and VII-15: 0.72. Betaine HCl: III-11, III-2, and III-3: 0.30; III-5: 0.10; III-4: 0.20; IV-4: 0.81; VII-16: 0.60.

DL-isoleucine 0.5%, glycine 0.5%, DL-threonine 1.0%, and DL-leucine 2.0%. The protein was reduced in amount equal to the amino acids added.

The supplements added to the basal diet are shown in Table I, columns 2-4, and the notes. The 0.50% cystine used was considered moderately in excess of the requirement for this amino acid (6).

## RESULTS

The growths obtained (as pen averages of the daily percentage increase in average weight), and such growths adjusted to a standard



initial weight of 100 g., are shown in columns 5, 6 and 7 of Table I. The adjustment to standard initial weight is made by means of the regression coefficient 0.0175% decrease in growth/g. increase in initial weight (3). Section A of the table shows the effect on growth of various concentrations of choline and methionine, section C the effect of adding betaine to certain of these diets, and section B the effect of certain other additions and omissions. Section D shows the growth of control pens fed the practical rearing diet during the test period and illustrates the reproducibility of growth that is obtainable by the procedure used.

## DISCUSSION

### 1. *The Fitting of a Response Surface to the Data*

In growth studies it is usual to hold all the dietary factors constant except one. The results are then quite simply representable by plotting growth against the change in the variable factor, expressed either as concentration in the diet or as amount consumed. When it is attempted to study the effect of simultaneous change in two nutritional variables, however, the complexity of the problem and the difficulty of graphically representing the results is greatly increased. But so also is the information that the results are capable of yielding. In the present case three rectangular coordinates have been used to represent the simultaneous change in choline, methionine and growth. A three-dimensional space figure is then required to completely represent the results. But, since only flat surfaces are available, three two-dimensional cross-sections of the space figure, one in each of the three space planes have been used instead. If profiles are cut out on the solid figure by vertical planes at constant choline-Cl levels these will show the effect on growth of varying the methionine concentration alone. Profiles cut on the solid surface by vertical planes at constant methionine levels will show the effect on growth of varying the choline-Cl concentration alone. It should be noted that where profiles from the two sets cross, they must intersect. Thus the profiles from the two sets are mutually consistent. Contours cut out by horizontal planes at constant growth levels will show the mutual concentrations of choline-Cl and methionine required to sustain such growths.

The profiles showing the effect on growth of varying choline-Cl at constant methionine levels are shown in Fig. 1A and those showing the

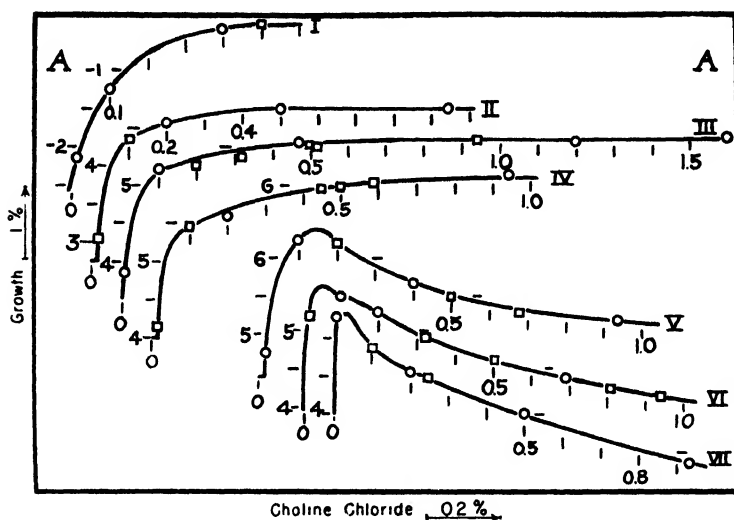


FIG. 1A. Vertical Profiles. Effects of varying choline-Cl at constant methionine concentrations. Profile Nos. and methionine concentrations: I: 0.14%; II: 0.30%; III: 0.40%; IV: 0.50%; V: 0.70%; VI: 1.00%; VII: 1.28%.

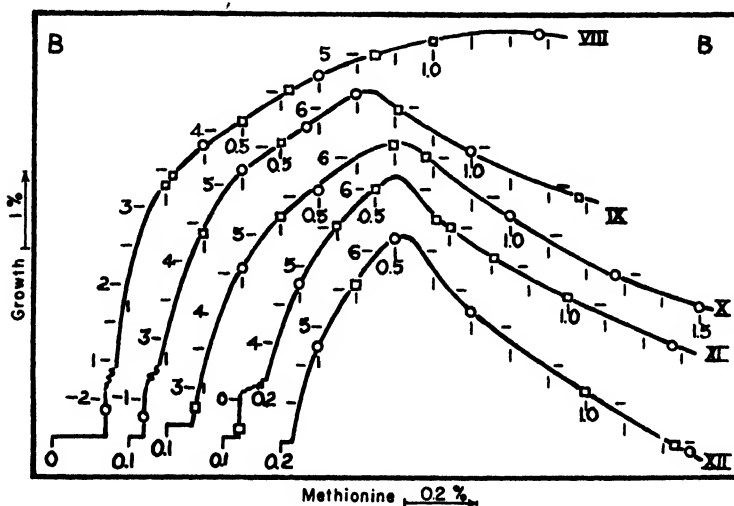


FIG. 1B. Horizontal Profiles. Effects of varying methionine at constant choline-Cl concentrations. Profile Nos. and choline-Cl concentrations: VIII: 0.015%; IX: 0.10%; X: 0.20%; XI: 0.50%; XII: 0.94%.

effect of varying methionine at constant choline-Cl levels in Fig. 1B. These curves have been constructed from the data in Table 1, section A. In these figures the circles represent the experimental points and the squares represent the intersections which the appropriate profiles from the two sets would have in space. In drawing the profiles the reasonable limitation has been imposed that curves will be smooth and that abrupt changes in curvature, except where maxima occur, are not to be expected. The effect of the mutual consistency of the two sets of profiles in delimiting the 3-dimensional surface upon which the contours are based should be clearly realized. Trial will show that in most cases it is not possible to raise or lower any of the square points on one profile, which represents the intersection of a curve from each set, more than about 0.1% in growth without unreasonably distorting the corresponding profile in the other set. To save space and prevent overlapping these profiles have not been drawn with reference to the same origin, but the growth and choline-Cl or methionine concentrations are marked along the individual curves. By reading from these experimental curves the various mutual concentrations of choline-Cl and methionine required at several levels of constant growths, contours showing this interrelation have been constructed. These are shown in Fig. 2. The small points are those taken from the curves in Figs. 1A and 1B; it is through these that the contours have been drawn; the experimental points are shown as larger circles. This figure may be considered as the horizontal base upon which the 3-dimensional solid would rest and the curves as the shadows cast on this base by the contours in the space model. The dashed lines (horizontal and vertical) then show the intersection with this base of the vertical planes that yield the profiles in Figs. 1A and 1B. (The diagonal dashed lines represent the traces of what would be diagonal vertical planes in the space figure along which both choline chloride and methionine vary. These 6 diagonal profiles (Nos. XIII to XVIII), though not reproduced, were also used in constructing the contours and were of great utility in establishing within narrow limits the mutual consistency of all the curves in the three sets (Figs. 1A, 1B and 2)). The Roman numerals in Fig. 2 show which dashed lines correspond to which profiles in Fig. 1. It should be noted that the profile curves (Fig. 1) are plotted directly from the experimental data. The contour curves (Fig. 2) are cross-plotted from the profiles. Their accuracy, relative to Fig. 1, is then limited only by such small deviations from mutual consistency

as may exist among the two sets of profiles and the accuracy with which interpolations can be made on smooth curves.

It will be noted that in Fig. 2 the experimental points tend to concentrate in the lower left corner of the figure. It is therefore in this part that the possible deviation of the contours from the indicated

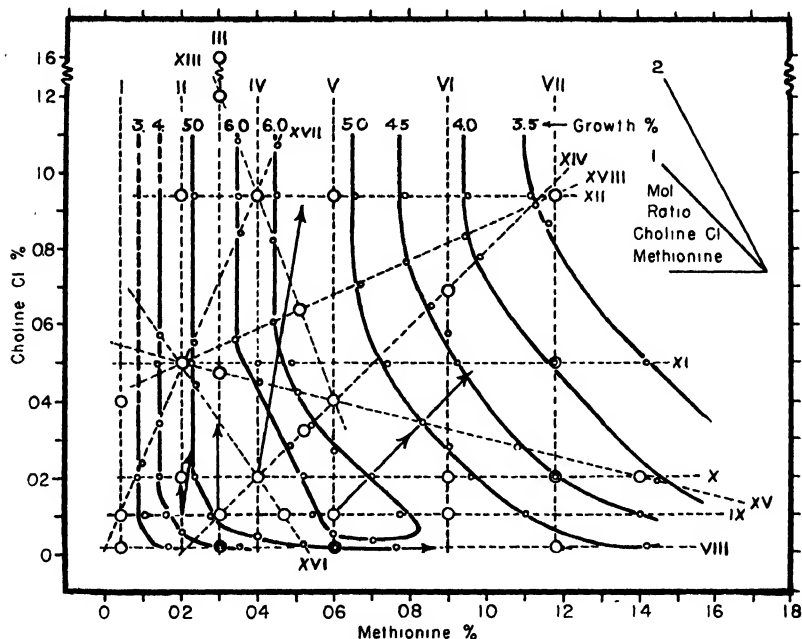


FIG. 2. Growth contours: mutual concentrations of choline-Cl and methionine required to produce constant growth. (Note: This figure is analogous to a contour map in which growth replaces elevation above the datum plane and choline and methionine concentrations replace distances north and east.)

locations is least. But the upper part of the figure is very important and it will be well to consider what additional facts there are to substantiate the locations chosen for the contours on this region: Profiles II and III (Fig. 1A) are exactly horizontal in their upper parts so that interpolations along these curves are as good as experimental points. Profile I, within the limits of the experimental points, is approaching the horizontal, and evidence will be brought forward to show that it should become horizontal in correspondence with profiles II and III. Extrapolation of this curve cannot then be in doubt more than about 0.1%

in growth. Thus the upper parts of the contours on the left can be considered to be as precise as their lower parts. The upper right of the figure is sustained by profiles XI and XII and by diagonal profiles XIII, XIV and XVIII (not reproduced). The upper half of diagonal profile XIV is well sustained by four experimental points. (The terminal point on profile XII is so close it may also be considered as on profile XIV.) Diagonal profile XVIII has only 2 experimental points on its upper half, but the intersection with profile V gives an interpolation which, because of the small slope of the latter curve (see Fig. 1A), is quite accurate. The intersection with profile IV is where the latter is horizontal. Thus, there are essentially 4 points determining the upper half of profile XVIII. Diagonal profile XIII has only 3 experimental points on its lower part, but the profile is short. Profile XI (Fig. 1B) has only 2 experimental points, but it intersects profiles III and IV where they are horizontal and profiles V and VI where the slope is quite small. (On profile V it intersects only the extrapolated part of the curve, but the extrapolation is quite short.) Profile XI may then be considered to be fairly well established. Profile XII is very important, but its right half has only 3 experimental points; still, these are fairly well placed and the curve evidently has the same form as Profile XI. There is an additional point on its left part and it intersects profile III where the latter is horizontal. The upper right contours are thus fairly well located also. The form of the tips of the contours in the lower right corner is not well established, but no conclusions are based on this part of the figure.

## *2. Response to and Interrelations of Choline and Methionine*

Consider now the characteristics of the contours in Fig. 2. The nearly horizontal parts show the limits beyond which choline cannot be decreased if growth is to be maintained, irrespective of the methionine concentration. These amounts (called the essential choline (4)) are presumably used in tissue formation. There is a suggestion in these parts, particularly along the 5% contour, that increasing the methionine to high levels slightly decreases the essential choline needed. This may indicate synthesis of small amounts of choline from certain of its decomposition products and is not unlikely since it has recently been found that, in the chick, dimethylaminoethanol in the presence of methionine will permit some growth (7). The essential choline required is about the same as has been found to be needed by incipiently perotic

birds, but greater than needed by highly perotic birds (4). The requirement for optimum growth (*i.e.*, over 6%) appears to be about 0.1%. For a growth of 5% or less the requirement is about 0.01% or less.

The nearly vertical parts of the curves at the left of Fig. 2 show the methionine needed to sustain the growths indicated, irrespective of increase in choline. These amounts, to be called essential methionine, are presumably used in tissue formation and in any reactions in which methionine is destroyed.

The diagonal curves connecting the vertical and horizontal parts at the left and bottom of the figure show the range in which choline and methionine are mutually replaceable. These amounts are presumably used in transmethylation. The slope shown, which seems to best represent the experimental results, is such that 2 moles of choline replace one of methionine. It should be pointed out, however, that there are not enough experimental points to make this ratio certain. Indeed there is no reason why the ratio should be any whole number. The replaceability of methionine by choline must mean either that choline methylates the ultimate acceptors or that synthesis of methionine from choline and the residues of methylation is rapid. When the methionine is near the essential level each molecule of residue must be remethylated many times. If the residue is homocystine this might be expected, since homocystine has not been found in tissue, and, if formed, must be rapidly removed. The contours then lead to the conclusion that there are no unique concentrations of choline and methionine which will sustain a given growth, but that each must have a certain level (its essential concentration) and that there must also be an additional concentration (to provide methylating capacity) which can be supplied by either.

Examination of the profiles (Fig. 1B) or of the contours (Fig. 2) shows the important fact that more than about 0.8% total methionine (*i.e.*, essential and replaceable) in the presence of adequate essential choline has a depressing effect on growth (see profile IX, Fig. 1B, or the contours crossed by line IX, Fig. 2). More than about 0.5% essential methionine in the presence of adequate total choline (*i.e.*, essential and replaceable) also depresses growth (see profiles XI and XII, Fig. 1B, or the contours crossed by lines XI and XII, Fig. 2).

Examination of profiles I to IV (Fig. 1A) or lines I to IV (Fig. 2) shows, however, that high concentrations of choline in the presence of

less than the essential methionine needed for optimum growth (0.50%) have no depressing effect. This is well sustained by profiles III and IV, particularly III, along which growths of 5.56%, 5.56% and 5.62% were obtained with choline-Cl concentrations of 0.47%, 1.20% and 1.60%. Profiles V, VI and VII (Fig. 1A) or lines V, VI and VII (Fig. 2) show clearly that, in the presence of more methionine than that essential for optimum growth (0.50%), increasing the choline does decrease growth. (It should be noted that the horizontal form of the upper parts of profiles II, III, and IV, and the fact that profiles I, V, VI and VII are rapidly approaching the horizontal, establishes definitely the vertical form of the contours in the upper part of Fig. 2.) As far as can be seen from the profiles in Fig. 1B optimum growth is the same for the various mixtures of choline and methionine that give the maxima of the curves (provided choline-Cl is above the concentration essential for optimum growth—about 0.1%); that is, the maxima of profiles IX to XII are the same and only the maximum of VIII appears to be lower.

The contours on the right side of Fig. 2 (corresponding to the right half of the profiles in Fig. 1B) show the mutual levels of choline and methionine required to produce a constant depression in growth. These contours have several characteristics that should be noted. First, at the lower choline concentrations they are sloping, showing that choline and methionine are here mutually replaceable. The slope is best represented by one choline molecule replacing one methionine. Secondly, the methionine required to depress growth is only partly replaceable by choline as shown by the change of the diagonal parts of the curves to vertical at higher choline levels. This indicates that the depression of growth by methionine has at least two causes, as does also its stimulation, where methionine functions as a tissue constituent and as a methylating agent. Choline in excess of that which can replace methionine has no effect on growth (again shown by the existence of the vertical parts of the curves). These facts indicate that methionine is the direct methylating agent and that choline only methylates methionine residues.

There are no profiles in Fig. 1B, between 0.015% and 0.10% choline-Cl (profiles VIII and IX), to show the precise form of the tips of the contours in Fig. 2. The fact that the tips of the contours cannot lie at a choline-Cl concentration much above 0.1% does show, however, that there is no great difference in the concentration of choline essential to sustain depressed growth as compared to stimulated growth. Probably

there is no difference at all, a further indication that depression is caused by methionine.

### 3. Interpretation of the Growth Response to Methionine in Terms of a First Order Reaction and of the Michaelis-Menten Equation

In Fig. 3 (upper curve) the logarithms of the essential methionine required to stimulate and to depress growth are plotted against the growth. The values used, plotted as circles, are the values given by the

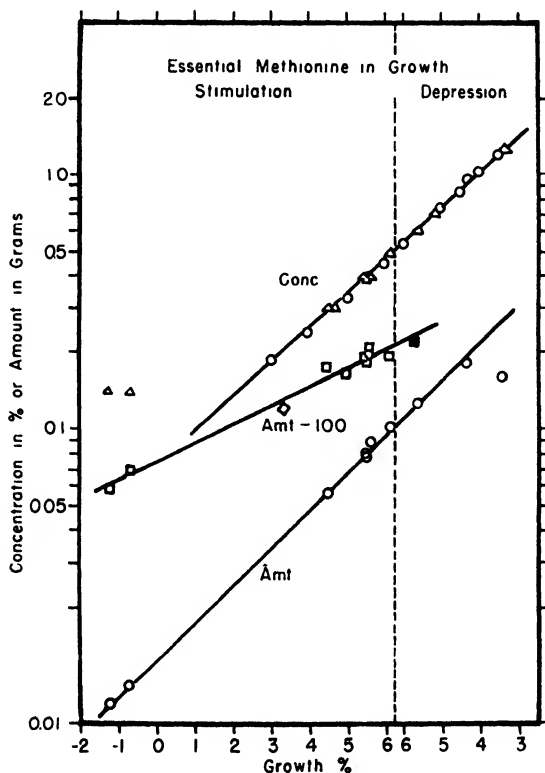


FIG. 3. Logarithmic relation of essential methionine and of food consumption to growth stimulation and depression.

vertical parts of the contours in Fig. 2. The curve is found to be a straight line, the slope of which is the same in both stimulated and depressed growth. It may be considered that this curve is somewhat



hypothetical since it is not based on direct experimental values, but on the contours in Fig. 2, which are themselves cross-plotted from Fig. 1. Fortunately, a test of its validity is available. If the contours in Fig. 2 really reflect experimental fact, and if the interpretation placed upon them is correct, then any experimental points lying in the region where the contours are vertical should, like them, represent the methionine essential to produce the growths found experimentally and so should lie on the curve in Fig. 3. Twelve such points are available: the two upper points on line I; the three upper points on each of lines II and III (Fig. 2); the upper points on lines IV, V, VI, and VII, and the middle point on diagonal line XVIII. These points are plotted in Fig. 3 as triangles. They fit the curve well, which not only lends support to the curve itself, but to the contours upon which it was based. The only points falling off are at a methionine concentration of 0.14% where there is no growth, but a loss in weight.

The equation for a first order reaction gives a straight line in which  $\log C = K_1 t$  ( $C$ , concentration of reactant;  $t$ , time). Now during the fourth week of life the growth of the chick is constant, within the limits of experimental error; earlier and later it departs from this linearity (8). In this period of time, then,  $X$  (growth)  $= K_2 t$ . Substituting then:  $X = K \log C$ . Fig. 3 then indicates that growth, at least within these experimental limits behaves as though governed by a first order reaction, in which the concentration of reactant in the food can be used instead of its concentration in the reaction system, *i.e.*, the tissues. Nor is it unreasonable that, when growth is limited by lack or superfluity of only one dietary constituent, it should follow the course of a pseudomonomolecular reaction.

Some interesting suggestions can be obtained by plotting some of the functions derived from the Michaelis-Menten equation for the velocity of an enzyme reaction (9). Under the condition that only one dietary ingredient is deficient, and has only a single function in metabolism, a single enzyme reaction may quite possibly control growth, which can then be taken as a measure of the velocity of such reaction. These conditions should be satisfied when methionine is reduced to its essential level and all methylating requirements are supplied by choline. Since, as already explained, the evidence indicates that the concentration of dietary methionine can be taken as a measure of its concentration in the tissue, and so in an enzyme system, the levels of methionine essential to support the growths found experimentally may be taken

from the curve in Fig. 3, which has itself been shown to agree with the experimental points as well as the contours upon which it was originally based.

The depression in growth caused by high concentrations of methionine may be the result of the formation of an inactive enzyme-substrate compound according to the reaction  $E + nS \rightarrow ES$  (active) +  $ES_{n-1}$  (inactive). The equation for the velocity of this reaction can be written (9)

$$\log \left( \frac{[S]}{V} - \frac{+K_s}{V_m} - \frac{-[S]}{V_m} \right) = n \log[S] - K_2 V_m$$

where  $E$  = enzyme,  $V$  = velocity (here percentage growth),  $S$  = substrate (here methionine);  $K_s = [ES]/[E] \times [S]$ ;  $V_m$  = maximum velocity, *i.e.*, velocity if no depressing reaction occurred;  $K_2 = (ES_{n-1})/[E][S_{n-1}]$ . As usual a bracket about a symbol indicates concentration in moles/l. of solution.

For this case a plot of  $\frac{[S]}{V}$  vs.  $[S]$  should give a straight line at the lower concentrations of  $S$  (9). This is found to be so. The slope and intercept of this line give values from which  $K_s$  and  $V_m$  can be calculated. The latter is found to be 14.7% (in terms of growth).

A plot of the left hand member of the velocity equation against  $\log [S]$  should also give a straight line. Trial shows that a straight line is obtained over the range corresponding to depressed growth, but, in the range corresponding to growth stimulation, the logarithm became indefinite owing to the approximate equality of  $\frac{[S]}{V}$  and  $\frac{K_s + [S]}{V_m}$  and the relatively low accuracy of the work for calculations of this kind. The slope of the straight line part of the curve, however, gives the reasonable value of 3.1 for  $n$  in the velocity equation.

If the reaction mechanism is simply:  $E + S \rightarrow ES$  (active), a plot of  $\frac{1}{V}$  vs.  $\frac{1}{[S]}$  should give a straight line (9). This is found to be the case in the range of growth stimulation. The combined results suggest the formation of inappreciable amounts of inactive compound during growth stimulation and a much more rapid formation during growth depression.

Using the constants calculated from the slopes and intercepts of the curves just discussed, a theoretical curve for the relation of growth to the concentration of essential methionine in the diet has been calculated. This curve is plotted in Fig. 4 as a solid line. The values for essential methionine derived from the vertical parts of the contours in Fig. 2 and the 10 experimental points in that region of Fig. 2 (*i.e.*, the same points

that were plotted in Fig. 3) are indicated along the dotted curve. The curve is a good fit at the lower growth values, but falls below the experimental values where growth is highest, which presumably indicates inaccuracy in deriving the constants for the curve. Comparison with profiles IX or X (Fig. 1B) or with the dotted curve (Fig. 4), however,

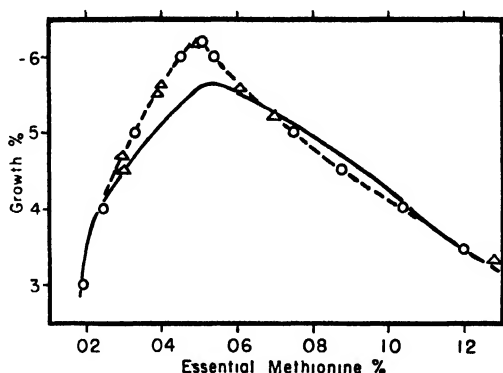


FIG. 4. Relation of growth to concentration of essential methionine. Points: experimental values; solid curve: values calculated, assuming growth to be stimulated and depressed by the formation of active and inactive enzyme-substrate compounds.

shows the interesting fact that both calculated and experimental profiles have the same form. In particular, the same rapid rise from lower growth levels, the presence of a shoulder, the almost straight line increase at higher growths, and the almost straight line falling off in growth.

The good agreement, in general, between experimental and calculated values lend support to the interpretation that stimulated growth is here limited by one rate-governing enzyme reaction and that this growth is depressed, not by overproduction of any metabolite, but by the formation of an inactive enzyme-substrate compound in the same system. The fitting of theoretical curves to the concentrations of essential methionine does not, however, prove that the reactions are just those suggested.

#### 4. Food and Methionine Consumption

It is interesting, but not surprising, that the simple relations developed in Figs. 2 and 3 can be based on the concentration of dietary

ingredients in the food rather than in the tissues. The fact that chicks respond to changes in the amino acid (or choline) content of their diets within a few hours suggests that there is little, if any, storage of these materials, so that concentration in the tissues must be closely proportional to the amount eaten. That this is so is shown by certain of the experimental results, but first, the variation in food consumption with growth may be considered briefly. Variation in food consumption cannot be as accurately measured as growth because only pen averages including occasional abnormal birds are available. The variations found in the data, however, are probably not due entirely to the use of pen averages, but arise also from the fact that more than one variable in the diet is changing (*i.e.*, both choline and methionine in both their essential and replaceable functions). When only one dietary variable is changing the variation is less. In Fig. 3 (middle curve) the logs of the amounts of food consumed, in g./chick/day (divided by 100 for convenience in plotting), are plotted against growth for 10 of the 12 experimental points shown in the upper curve in which methionine is at its essential level. The squares are points for stimulated growth and the diamonds for depressed growth. The solid point represents the growth and food consumption obtained with the practical rearing diet used in the control pens (Table I, Section D). For this curve, stimulated and depressed growth are both represented on the left part of the abscissa. The points vary from the curve by less than  $\pm 7\%$ . All the other experiments in Table I in which other variables come into play have been tried on this curve and form a fringe on both sides in which the maximum variation is about  $\pm 20\%$ . It is interesting to note that even where there is loss of weight the points are still on the curve.

In Fig. 3 (lower curve) the amounts of methionine consumed, in g./chick/day (calculated from the pen averages of food consumed), are plotted for 10 of the 12 experimental points that are plotted as triangles in the upper concentration curve. These points also give a straight line almost exactly parallel to the concentration curve. This parallelism means that there is a direct proportionality between the concentration of methionine in the diet and the amount consumed and consequently between the concentration in food and tissues. This finding justifies the use of dietary concentrations in the analysis of the results. The relation also implies that at any growth the food consumed is the same in both stimulated and depressed growth, as is also indicated by the curve for total food consumed.

All the experiments in Table I have been tried on the crures for the concentration of essential methionine in the diet and for the amount of essential methionine consumed, and in all cases these points form a fringe lying entirely above the curves. This simply means, of course, that when methionine has more than one function to perform, more is required.

It may be noted that the two points at low methionine concentration and negative growth that fall above the concentration curve are on the curve for methionine consumed. This shows that experimental error was not here involved. The probable explanation is that there is a threshold concentration of methionine required, and that when it falls below this value there is a rapid falling off in both food and methionine consumption and, consequently, in growth.

But the question may possibly arise: Is growth depression not the result of insufficient food? But here it should be noted that during both growth stimulation and depression methionine consumed increases with concentration in the diet, although food consumed decreases, so that the gross concentration of methionine in the bird also increases. For the points taken as a whole, where the functions of choline and methionine vary, *i.e.*, where they may function as tissue constituents or as methylating agents, the rate of increase in methionine consumed with methionine concentration is still approximately constant. For the points plotted in Fig. 3, where methionine is at its essential level and has only one function while choline is in excess, and points fit a straight line very closely. Since the only causal factor for both decreased food consumption and decreased growth is the increased concentration of methionine or choline in the body, it is illogical to consider decreased food consumption the primary cause of decreased growth. Decreased food consumption appears to be caused by decreased physiological need, itself caused by the inhibitory effects of the higher concentrations of methionine. It is, then, an effect of decreased growth and not a cause. Indeed, if food consumption were not decreased, the excess food would have to appear in the feces, the urine, or as an increase in metabolic rate. This is in agreement with the fact that, when the methionine concentration is so low that the straight line relation between concentration and growth no longer holds, the food consumed still seems to be determined by the methionine consumed.

If, as suggested above, food is consumed only in so far as it can be used, the limit must be determined by the rate at which the enzyme

systems can transform their substrates into the products of their syntheses. These rates are determined by the amount of enzyme present and by the concentration of substrate. This last may be either too low or too high so that the enzyme either is not all in the form of active enzyme-substrate compound or is partly combined as higher inactive enzyme-substrate compound. Now the simplest explanation of the cause of the stimulus that prevents eating would be that it arises from the presence of too high a concentration of some metabolite in the blood. So for example, if food consumption (and growth) is low because of too low a concentration of methionine in the diet it might be the concentration of one of the other essential amino acids that limits food intake. On the other hand if food consumption is low because of too high a concentration of methionine in the diet it might be the methionine itself that limits consumption. But this is unlikely for the reason that food consumption is the same in both stimulated and depressed growth and because the excess methionine is required to form enzyme-substrate inactive compound. The mechanism is more likely to be the same as in stimulated growth. If these assumptions are correct, the protein consumed in the food should be divisible into two increments: one going to form tissue and the other providing the inhibitory amino acids in the blood. This second increment should be constant at all growths. Now such a value can be calculated from the available data as follows:

The overall methionine content of chick tissues is close to 3.0% and, since the composition of tissues is constant, dividing the methionine consumed (taken from the curve in Fig. 3) by this value should give the protein formed, after subtracting protein equivalent to the methionine consumed when growth is zero (0.49 g.). Twenty per cent of the food consumed (taken from the curve in Fig. 3) gives the protein consumed. The difference (shown in column 4, Table II) represents the increment that is assumed to inhibit food consumption and this, as suggested, is constant. This value may be independently calculated in another way: the normal 3-weeks old chick contains 18.0% protein and the normal 4-weeks bird 19.5% (10). It may be assumed that progress from the lower to the higher value will be proportional to the relation of actual growth to normal growth. From these values and weights and growths of the birds given in Table I the protein deposited can be calculated. Subtracting these values from the protein intake gives the values in column 6 of Table II.

The agreement in "excess protein" calculated by the two methods is quite satisfactory except for the value at 6% growth calculated from increase in weight.

TABLE II

Stimulated Growth	Protein Consumed	Protein Anabolized	'Excess' Protein	Protein Anabolized	'Excess' Protein
		(Calculated from Methionine Consumed)		(Calculated from Increase in Weight)	
<i>per cent</i>	<i>g.</i>	<i>g.</i>	<i>g.</i>	<i>g.</i>	<i>g.</i>
-1.0	1.4	-0.1	1.5	-0.2	1.6
0	1.6	0.0	1.6	0.0	1.6
+1.0	1.9	0.2	1.7	0.5	1.4
2.0	2.2	0.4	1.8	0.8	1.4
3.0	2.6	0.7	1.9	0.9	1.7
4.0	3.0	1.2	1.8	1.3	1.7
5.0	3.6	1.8	1.8	1.7	1.9
6.0	4.4	2.7	1.7	2.1	2.3
Mean			1.7		1.8

5. *Effects of Serine, Glycocyamine and Homocystine on the Response to Methionine.*

That methionine depresses growth of itself is also indicated by experiments in which the composition of the diet used in Exp. V-2 (0.20% choline-Cl and 1.50% methionine) was changed by additions or omissions. This diet gave a growth of 4.14%. The methionine is equivalent to 10.1 mM/100 g. diet. In Exp. V-4, 4.9 mM glycocyamine (0.58%) were added, increasing the growth to 5.26%. Since glycocyamine increases muscle creatine in the chick (11) it presumably increases growth here by providing an acceptor for the methyl group of methionine. In Exp. V-5, 4.9 mM serine (0.52%) were added, which increased the growth to 4.96%. Since (in the rat) serine furnishes the carbon chain for cystine synthesis (12) and methionine the sulfur atom (13), the experiment may indicate that providing additional serine promotes cystine formation and uses up methionine. But serine is also easily decarboxylated and the amino group may act as a methyl acceptor. Since the chick can provide serine for its own tissues (12), free serine is apparently required. While no analyses for the serine of soybean protein have been found, the amino acid is probably present and the effectiveness of the supplement is presumably a matter of concentration.

Omitting cystine from the diet might conceivably promote its formation from methionine, but this does not turn out to be so, since omitting the 0.50% cystine supplement (Exp. V-3) still further decreased growth to 3.63%. Adding 0.52% serine to this diet (Exp. V-6) increased the growth from this level to 4.33%, almost the same increment of increase as was obtained when serine was added to the diet containing cystine. Doubling the glycocyamine and serine (Exps. VII-13 and VII-12) also increased the growth, but less than the smaller amounts. Presumably the reactions went too far and the methionine available was less than that required for higher growth.

A diet containing 0.20% choline-Cl and 0.70% methionine would be expected from Fig. 2 to give a growth of about 6.0%. In Exp. VII-14 0.72% homocystine added to such a diet reduced the growth, but only moderately, to 5.27%. This reduction may indicate some synthesis of methionine from homocystine and the 0.20% choline-Cl, or it may indicate excessive synthesis of cystine (to be considered in a later paper). Increasing the choline-Cl to 1.30% (Exp. VII-15) further decreased growth to 3.98%. From Fig. 2, 1.30% choline-Cl and 0.70% methionine in the absence of homocystine would be expected to give a growth of about 5.3%. This pair of experiments shows that homocystine in combination with methionine does not greatly depress growth unless additional methyl groups are provided to promote synthesis of further methionine.

### 6. *The Role of Glycine Betaine*

The available evidence goes to show that betaine, in the chick, functions as a methylating agent (4, 14) and, from the results just presented, it would be expected that, when used to supplement choline or methionine, it would stimulate or depress growth depending on whether additional methylating capacity was needed or was undesirable. If, however, growth were limited by lack of essential choline or methionine, the addition of betaine should be without effect. These conclusions are borne out by the experiments in Table I, Section C, the results of which are plotted in Fig. 2 in the following way: From the point corresponding to the choline and methionine concentrations of the diet an arrow (vector) is laid off whose length corresponds to the betaine HCl in the diet and whose direction is such that it terminates at a point in the field corresponding to the growth found experiment-



ally. It is theoretically possible that there may be points in the field from which more than one vector satisfying these conditions could be laid off, but experimentally only one vector has been found that will satisfy the conditions of each experiment. A vertical vector would indicate that betaine supplemented the effective choline content of the diet but did not supplement the effective methionine content; a horizontal vector, that it added to the effective methionine content but did not affect the effective choline content. While Fig. 3 is plotted on a weight basis, the molecular weights of choline-Cl (140) and methionine (149) are nearly enough the same so that the plot represents quite fairly the molar relations. The molecular weight of betaine HCl (155) is, however, about 5-10% greater and the vectors have been correspondingly shortened. While it has been shown that with chicks raised on a low choline diet about 1.25 moles of betaine replace one of choline (4), the mol ratios by which betaine replaces choline or methionine in chicks raised on an adequate diet are not known. But, although the vectors should be shortened by amounts corresponding to any excess of such ratios over one, it will be found that this source of uncertainty does not affect the conclusions.

In Exp. III-14 (0.015% choline Cl, 0.70% methionine) growth was 4.71%; adding 0.30% betaine HCl to this diet (Exp. III-11) only increased growth to 4.90%. Here Fig. 3 shows growth to be limited by lack of essential choline, the methionine being sufficient for both the essential and replaceable increments. This appears from the contours which show that increasing choline would increase growth, but that increasing methionine would not. The effect of adding betaine should then be the same as adding more methionine, *i.e.*, a still further increase in methylating capacity. In agreement with this analysis the direction of the only vector that will indicate the experimental growth is found to be horizontal.

A diet containing 0.10% choline-Cl and 0.30% methionine should produce a growth of 4.45% (profile IX, Fig. 1B); adding 0.10% and 0.20% betaine HCl (Exps. III-5 and III-4) increases the growth to 4.75% and 4.99%. Here the contours in Fig. 3 show that there is adequate essential choline and some lack in replaceable choline but growth is mostly limited by lack of essential methionine. The vector might then be expected to be vertical indicating an increase in the apparent choline content; actually it tips a little to the right (this point will be discussed later), indicating in addition a slight sparing

action on essential methionine. Any excess of the ratio by which betaine replaces choline or methionine over one would simply shorten the vector and increase the tipping. Similarly Exp. III-7 (choline-Cl 0.10%, methionine 0.40%) gave a growth of 5.19%; adding 0.30 betaine HCl increased growth to 5.50%. In Exp. IV-1 (choline-Cl 0.20% and methionine 0.50%) growth was 5.55%; adding 0.81% betaine HCl only increased it to 5.62%. Here the vector shows that too much betaine has been added, carrying the point into the region of depressed growth. Judging from the contours, 0.20% betaine HCl would have been the optimum amount to add to this diet and would have increased the growth to 6.0%. Exp. III-8 (choline-Cl 0.10% and methionine 0.70%) gave the best growth obtained (6.21%). Here, adding 0.30% and 0.60% betaine HCl (Exps. III-3 and VII-16) carries the point into the area of depression and growth is depressed to 5.08% and 4.41%. Since choline and methionine are together adequate, or very nearly so, for both essential and replaceable requirements, betaine should add to the apparent content of both and, in agreement with this, it is found that the vectors are best drawn at 45°. This is also in agreement with the indication of the contours that, in depression of growth, choline and methionine replace each other mole for mole.

It has been shown (4) that betaine sometimes increases growth more than an equivalent amount of choline and the explanation offered was that choline may methylate the methionine residue reversibly, but that in the case of betaine the reaction might be irreversible. The earlier experiments were in a range where total choline was adequate but essential methionine inadequate. The same phenomenon is found in the present case and is indicated by a tipping of the vectors to the right, showing that the addition of betaine increases the effective concentration of essential methionine.

An interesting comparison showing the major effects of physiological condition on nutritional requirements may be made between the results of the present experiments on normal chicks and those on the very perotic chicks previously reported (4). In the latter birds, 0.30% methionine and 0.03–0.04% choline-Cl with about 0.20% betaine HCl, for additional methylating capacity, was sufficient to give optimum growth (6.4%). These amounts of choline and methionine are, therefore, at least adequate for the essential needs. Since increasing the choline-Cl to 0.1% or over depressed growth, the 0.2% betaine HCl must represent most of the needed methylating capacity. The present

experiments (see Fig. 2) indicate that for maximum growth with normal birds the requirements for essential choline-Cl (about 0.1%) and for transmethylating capacity (about 0.3–0.4%) are about twice what they were in the perotic birds. The need for essential methionine in the normal birds (0.5%) is also much larger, since in the perotic birds 0.30% is beyond the optimum requirement, so that increasing the choline can depress growth.

#### ACKNOWLEDGMENTS

This work was aided by research grants from the Nutrition Foundation, Inc., and from Swift and Co.; biotin was supplied by Merek and Co., Inc., through the kindness of Dr. J. C. Keresztesy.

#### SUMMARY

1. The conclusions that follow are based on experiments with the normal White Leghorn chick during the fourth week of life.

2. The choline and methionine required in the stimulation of growth can be divided into two parts, which may be called the essential and the replaceable parts. The essential increments cannot replace each other, but the replaceable increments can. For optimum growth 0.50% methionine and 0.10% choline-Cl are essential, plus methionine (0.25%) or choline-Cl (0.45%) or any equivalent mixture.

3. Excessive methionine will depress growth and is divisible into an essential part and a part replaceable by choline. Choline-Cl (up to 1.60%), except as it can replace excessive methionine, does not depress growth. Within the limits studied, growth has been depressed from 6.21% to 3.35%.

4. The depressing effect of methionine can be counteracted by adding the methyl acceptor, glycocyamine, or by adding serine, which last may promote the formation of cystine, but omitting cystine itself depresses growth still further. High concentrations of homocystine do not depress growth much unless the choline needed to form methionine is present.

5. The experimental values for essential methionine, both in stimulation and depression of growth, can be fitted to a calculated velocity curve derived from the Michaelis-Menten equation for the velocity of an enzyme reaction in which both active and inactive enzyme-substrate compounds are formed. The relation between essential methionine and growth is that of a first order reaction.

6. Glycine betaine can substitute for the replaceable choline or methionine in both stimulation and depression of growth, but not for the essential increments.

7. The optimum growth obtained on an experimental diet, 6.21%, is to be compared with a maximum growth of 6.97% obtained under the same conditions using a practical rearing diet.

8. There is a straight line relationship between the log of food consumed and growth, irrespective of whether growth is stimulated or depressed.

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# LETTERS TO THE EDITORS

## Pteroylglutamic Acid (Folic Acid) and Neoarsphenamine Toxicity

Sirs:

It has been shown that some whole aqueous liver extracts are effective in preventing the acute toxicity of several drugs (sulfanilamide, diethylstilbestrol) (1) (neoarsphenamine) (2, 3). We thought that it would be of interest to know if pteroylglutamic acid (PGA), which is present in liver preparations, could be partly responsible for their antitoxic activity. However, unexpectedly we found that PGA when injected in mice is able to increase the toxicity of neoarsphenamine.

Fasting mice (19–20 g.) were injected subcutaneously with increasing dosages of synthetic PGA.<sup>1</sup> One hour later they were given the LD<sub>10</sub> dose of neoarsphenamine (180 mg./kg.).<sup>2</sup> After 16 hours the percentage mortality observed was much higher in these groups than in the controls injected only with neoarsphenamine.

No. of mice	PGA	Neoarsphenamine	Mortality ratio
	<i>mg./kg.</i>	<i>mg./kg.</i>	
20	75	180	1/20
20	150	180	6/20
20	225	180	16/20
20	300	180	20/20
20	—	180	2/20
20	—	280	16/20

Two other lots of 50 mice (10 for each dose) were selected for the determination of the LD<sub>50</sub>. One group was injected with 150 mg./kg.

<sup>1</sup> Pteroylglutamic acid in 5% sodium bicarbonate. We wish to express our thanks to the Lederle Laboratories, Inc., Pearl River, N. Y., for the generous gift of synthetic pteroylglutamic acid.

<sup>2</sup> Neoarsphenamine Squibb.

of PGA one hour before the toxic dose. Both groups were then given graded dosages of neoarsphenamine. The  $LD_{50}$  for the neoarsphenamine group was  $250 \pm 19$  mg./kg. whereas the PGA-neoarsphenamine group presented the  $LD_{50}$  of  $185 \pm 12$  mg./kg. (4). Microscopic sections of the liver and kidneys showed a similar pathological picture, although the PGA-treated animals presented more severe necrotic and hemorrhagic lesions in the kidneys.<sup>3</sup> In a control group of 30 mice treated with PGA alone (75–150 mg./kg.) no toxic effects were observed. This fact is in accordance with the low toxicity for PGA found by Harned *et al.* (5).

The knowledge that mice are more susceptible to the acute toxicity of neoarsphenamine when injected previously with PGA is of interest, since large doses are now used clinically and could, therefore, be harmful if neoarsphenamine is administered on the same occasion.

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June 12, 1947

### An Adenine-pentose-pyrophosphate from Plant Tissues \*

Sirs:

We have succeeded in isolating from oat seedlings the material responsible for the labile ( $\Delta 7'$ ) phosphorus of the barium-insoluble fraction, using a modification of the procedure of Needham (1) (designed to isolate the adenosine triphosphate (ATP) from muscle). The substance from the plant is related to the ATP of the animal but is not identical with it. The molar ratio of adenine to pentose to phosphorus in the plant compound is 1:1:2, but the available data on the split

\* We are indebted to Dr. A. Penna de Azevedo of the Pathology Division, Instituto Oswaldo Cruz, for the pathological examinations.

product (presented below) suggest that its molecular composition corresponds to two moles of adenine, two moles of pentose, and four of phosphorus. Half of the phosphorus is liberated in 7 minutes at 100°C. in *N* HCl or by the action of the potato pyrophosphatase (2). Adenine was identified and estimated by its ultraviolet absorption maximum at 260  $\mu$ . The pentose phosphate in the plant material is not the ribose-5-phosphate obtained from animal ATP. First, the rate of color development in the orcinol-pentose reaction (3) of the plant material (33% of maximum color in 7 minutes) most closely resembles that of arabinose-5-phosphate and is significantly different from that of the other known pentose phosphates (ribose-5-phosphate—74%; xylose-5-phosphate—44%; ribose-3-phosphate—45%; xylose-3-phosphate—24%). The ribose-5-phosphate of animal ATP gives a rate curve in the orcinol-pentose reaction identical with that of pure ribose-5-phosphate. Second, after removal of the labile phosphorus with the potato pyrophosphatase, the plant analogue of adenylic acid (2 moles of adenine, 2 of pentose, 2 of phosphorus) is not deaminated by the 5-adenylic acid deaminase obtained from rat muscle (4) which was active on the 5-adenylic acid obtained from animal ATP.

Purification with Lohmann's reagent causes a breakdown of the material indicating a greater lability than animal ATP. The products resulting from this breakdown are inorganic phosphate and a material, now barium-soluble, which contains 2 moles of adenine, 2 of pentose and 3 of phosphorus. One of these latter is a pyrophosphate link inasmuch as it is removed by *N* HCl (100°C.) in 7 minutes or by the action of the potato pyrophosphatase. This indicates that the original material is not adenosine diphosphate, but that it resembles, in this respect, the di-adenine polyphosphate obtained by Kiessling and Meyerhof (5) from yeast. The material remaining after the removal of the pyrophosphate has the pentose reactions previously noted, is not deaminated by the 5-adenylic acid deaminase and liberates 27% of its phosphorus in *N* HCl at 100°C. in one hour (ribose-5-phosphate—30%, xylose-5-phosphate—32%, arabinose-5-phosphate—24%, ribose-3-phosphate—99%), giving a curve characteristic of a pentose-5-phosphate.

The material isolated is physiologically active and has the ability to phosphorylate pyridoxal in the tyrosine decarboxylase system (6). A brief survey, based on the rate of color development in the orcinol-pentose reaction, indicates that this compound may be widely distributed in plants.



## ACKNOWLEDGMENT

This work was aided by grants from the American Philosophical Society and the American Cancer Society. We are indebted to the Federal Nutrition Laboratory and the Laboratory of Bacteriology, Cornell University, where the preliminary work on this problem was done. We are also indebted to Levene Memorial Collection, Rockefeller Institute, for a supply of arabinose-5-phosphate and xylose-5-phosphate.

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Brooklyn College,  
Brooklyn, New York  
July 3, 1947

H. G. ALBAUM  
M. OGUR

## Incorporation of C<sup>14</sup>-Labeled Glycine into Intestinal Tissue and its Inhibition by Azide \*

Dear Sir:

Recently the uptake of S<sup>35</sup>-labeled methionine into rat liver slices was described (1). In the experiments outlined in the present communication, radioactive glycine, labeled with C<sup>14</sup> in the carboxyl group, was used to study the incorporation of glycine into the proteins of intestinal tissue sections. Intestinal mucosa has been found to be highly active in protein synthesis *in vivo* (2). Also, sections of rat intestines are easily prepared.

In addition to heat and mechanical disintegration of cells, a chemical agent, sodium azide, was found to be an effective inhibitor of glycine uptake by intestinal tissue. The following table illustrates the type of results obtained.

Treatment prior to incubation	C <sup>14</sup> concentration in counts/ min./mg. dry protein
None	1.3
Boiling	0.0
Homogenizing	0.0
2 × 10 <sup>-3</sup> M NaN <sub>3</sub>	0.1

\* Aided by grants from the American Cancer Society (recommended by the Committee on Growth of the National Research Council) and the Rockefeller Foundation.

Approximately 0.7 g. of tissue (cut into about 10 squares) was incubated at 36°C. for 90 minutes in 10 ml. of Krebs-Henseleit medium (pH 7.4) containing 0.1% added glucose and 4 mg. of radio-glycine, in a 95% oxygen-5% carbon dioxide atmosphere. Subsequently the proteins were precipitated by homogenizing with trichloroacetic acid and washed thoroughly with trichloroacetic acid and then with acetone.

The results suggest that the presence of radioactive glycine in the proteins of the untreated tissue is due to an incorporation into protein molecules, under the influence of enzymes. While sodium azide is considered to be a characteristic inhibitor of certain oxidative enzymes (*e.g.*, cytochrome oxidase), the possibility remains that it may have a more direct action on the enzymes which promote peptide bond formation.

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*Medical School, Division of Biochemistry,  
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July 31, 1947.*

THEODORE WINNICK  
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## Book Reviews

**Tabulae Biologicae. Vol. XXI. Digestion. Part 1.** General Editors: V. J. KONINGSBERGER, E. J. SLIJPER, AND H. J. VONK. Part 1 edited by H. J. VONK, J. J. MANSOURBEK, AND E. J. SLIJPER. Dr. W. Junk, Amsterdam, 1946. xvi + 284 pp.

If the results of scientific work are to be integrated, correlated, and applied, it is necessary that they be gathered together in a form which can be quickly and easily comprehended. *Tabulae Biologicae* constitute an assemblage of data from the general field of the biological sciences in tabular and graphic form, with sufficient textual comment to render the tables and diagrams intelligible. In this way, a relatively enormous amount of information can be included in small space. Critical evaluation is not attempted.

The section under review comprises data on the physiology of digestion, principally in the vertebrates. A second part on the same subject, and a third on the invertebrates are planned to complete volume 21. A few of the contributions in the present section were prepared before 1940, publication having been delayed by the war; an addendum to part 3 will include data from the intervening years. The work is all in the German language; most of the contributors are associated with the University of Utrecht.

E. J. Slijper presents a very valuable and extensive collection of information on the physiological anatomy of the digestive organs of vertebrates. Descriptive characteristics of the entire tract and of individual organs are clearly presented and numerical data are tabulated, giving lengths, weights, volumes and surface areas of the various portions, under normal and experimental conditions. A guide to families and orders of animals listed would have been helpful to the non-specialist here. The second article, by G. C. Hirsch, offers a short discussion of the theory of secretion, a classification of types of secretion and tabulation of examples, principally drawn from mammals, fishes, mollusks, and insects.

C. Romijn has tabulated in the third article the available information concerning the food of the vertebrates; the animals are arranged by class, order, and family. This extensive list should be of the greatest value to ecologists and comparative physiologists. There follows a brief tabulation, by B. J. Krijgsman, of mechanical factors in vertebrate digestion-intestinal movements and their appropriate stimuli, velocities of propagation, time for movement of food, and the forces involved. Information is included only for the common laboratory animals. Romijn has tabulated material on the rate of movement of food through the digestive tract of other animals, including several invertebrates. In the succeeding article, by the same author, data are presented on the chemical composition of the digestive organs of vertebrates; the lack of information on this subject is astonishing, when one considers how much these structures have been used in physiological experimentation.

Ch. Engel, in the seventh paper, includes a variety of information, in tabular form, on the digestive enzymes of vertebrates. A certain amount of textual comment would

have improved the usefulness of this section to readers unfamiliar with enzymology. Linderström-Lang and Holter give a series of graphs depicting the results of the work at the Carlsberg Laboratories on the distribution of enzymes in the intestinal mucosa of the pig. The concluding article, by H.P. Wolvekamp, tabulates the results of studies dealing with the composition and secretion of saliva in the vertebrates; descriptive and experimental studies are included.

The book is paper-bound, but well and clearly printed. There is no evidence of the fact that the contributors and editors did their work under conditions more trying than we can easily imagine. The succeeding parts of this volume will be awaited with interest.

BRADLEY T. SCHEER, Los Angeles, Calif.

**Practical Physiological Chemistry.** By PHILIP B. HAWK, President, Food Research Laboratories, BERNARD L. OSER, Director, Food Research Laboratories, and WILLIAM H. SUMMERSON, Associate Professor of Biochemistry, Cornell University Medical College. 12th Ed. Blakiston and Co., Philadelphia, Pa., 1947. xiv + 1323 pp. Price \$10.

To English-speaking students of biochemistry, "Hawk" is probably more familiar than any other laboratory manual. For years, "Hawk" for the laboratory and "Hammersten" for theory were to be found in the library of every biochemist. The 12th edition of Hawk, now before us, has as active co-authors Drs. Oser and Summer-son, and several others have been responsible for specific chapters: Sumner and Somers, enzymes; D. L. Thomson and Heard, hormones; Quick, putrefaction and detoxication; Block, proteins, amino acids and nucleoproteins; Karshan, teeth; and Jukes, folic acid.

It should be said at once that this voluminous text is, on the whole, well written and thoroughly up-to-date. The laboratory experiments are interspersed with sometimes brief, and sometimes more extended theoretical discussions. To some extent, inconsequential "little" experiments are still included; but, on the other hand, nothing seems to have been omitted; and what was true of the older editions is certainly true of this one: if you want particulars of a biochemical determination, examine Hawk first before hunting up the journals.

Now for some details. No, I do not like starting the book (Chap. I) with physico-chemical properties of solutions. It reminds me of the older texts in chemistry that devoted their opening chapter to physical chemistry and then completely forgot the subject in subsequent chapters. In this case, Chapter I is followed by carbohydrates (Chap. II) and there is no continuation whatsoever. I think a much better plan would have been to incorporate Chapter I with the proteins (Chaps. IV, V, VI), with which the contents of Chapter I have much in common.

Also, it is not clear to me why in Chapter III, which is headed "Fats" and has a subheading "lipids," we find a classification of lipids but a discussion of true fats only. We have to turn to Chapter XI, before lecithin and cholesterol are really discussed. Since we find lipids in every cell of the body, and since they do have common characteristics, why not include all three in the earlier chapter?

Again, I don't understand why proteins are followed by a chapter on milk, which, in turn, is followed by several chapters on tissues. We have to turn to Chapter XII

before we come to enzymes (a particularly good chapter, by the way). Here, too, it seems to me that since enzymes are such universal cell constituents, they should be treated in an earlier chapter—preferably following the proteins.

But these are matters of arrangement and not of content. From the point of view of content there is much to praise. Particularly appealing to me are the following discussions and experiments which accompany them: nucleic acid (p. 186); muscular contraction (p. 240); chemical changes in nervous activity (p. 258); phosphorylase (p. 289); cell respiration (p. 290); electrophoresis (p. 415); colorimetry and photometry (chap. 23); hormones (chap. 26); metabolism of acetic acid (p. 926); use of *Neurospora* mutants (p. 979); microbiological determinations (p. 976); isotopes (p. 987); antibiotics (chap. 36). The chapter on vitamins (chap. 35), as one might have anticipated, is the most complete thing of its kind I have yet seen.

Too little is devoted to the kinetics of enzyme action (p. 272); and too much, to gastric analysis (p. 330) (considering that "Hawk" is not a clinical book). The problem of proteolytic enzymes (p. 355) in the pancreas needs clarification—and this is not Hawk's fault. Likewise, what are biochemists going to do about those ill-defined acids and alkali metaproteins, proteoses and peptone (p. 180)? Is there no way of throwing them overboard? Formulae for bile pigments (p. 374), and several pages of sterol formulae (pages 687–690) are, in my experience, so many pages wasted, unless, of course, these are part of a course dealing with their chemistry. With the knowledge that some hormones are eliminated as sulfates and glucuronides, the concept of "detoxication" (p. 391) needs, perhaps, to be broadened. From my own experience, the conjugation of glucuronic with borneol is a highly instructive experiment (refer to p. 397).

It is easy enough to pick flaws. But let there be no misunderstanding: the Hawk, Oser and Summerson publication is an event of the first order of magnitude to every student of biochemistry. The biochemist will want his H., O. and S. side by side with his bunsen burner.

BENJAMIN HARROW, New York, N. Y.

**Essentials of Physiological Chemistry**, 3rd edition. By ARTHUR K. ANDERSON, Pennsylvania State College. John Wiley & Sons, Inc. 1947. vii + 395 pp. Price \$3.50.

This is the third edition of a textbook of biochemistry which was originally written to present the more important facts of biochemistry, as related to the animal body, in a form that will be understandable to a student with a limited preparation in chemistry and biology. The book is particularly useful, therefore, in a course in biochemistry for undergraduate students in biochemistry, home economics, premedicine, chemistry, bacteriology and agriculture.

In this new edition, many sections have been rewritten and others enlarged. A set of review questions has been added to each chapter to aid the student in evaluating his ability to remember factual material. Several important general references are included at the end of each chapter.

Professor Anderson's book will continue to be very useful for the purposes for which it was intended. It is written in a style which is direct and factual. The author is to be admired for his capacity to limit his consideration of the various topics to, in most cases, the most important and essential facts.

ABRAHAM WHITE, New Haven, Conn.

**Advances in Protein Chemistry, Vol. III.** By M. L. ANSON, Continental Foods, Hoboken, and JOHN T. EDSALL, Harvard Medical School, Boston. Academic Press Inc., New York, N. Y., 1947. xii + 524 pp. Price \$7.50.

The progress of modern science is marked by two conflicting tendencies. As the amount of material in the form of observed facts increases and the methods used are becoming more elaborate, a certain degree of specialization, both on the theoretical and experimental side, becomes unavoidable. On the other hand, there is an increasing tendency to develop fields which by tradition and custom are either a no-man's land or are claimed by two or more closely allied sciences. In fact, in many fields of science the traditional frontiers are disappearing fast. This situation is perhaps particularly marked in the field of protein chemistry, which has become a meeting ground for the physicist, physical chemist, organic chemist, biochemist, cytologist and physiologist. It is impossible for any one worker to master more than a few techniques, but it is clearly desirable that he should know what contributions other methods can make to the solution of his difficulties, and that he should be aware of the problems which arise in other, allied fields of protein chemistry. This need to inform the research worker of progress made in his own field and in branches of science closely allied to his own has been met in the case of protein chemistry very successfully by the publication of "Advances in Protein Chemistry," of which Volume III has just appeared.

Many of the contributions are apparently primarily addressed to the specialist, or at least to those chemists or biochemists with a particular interest in proteins. This applies, for instance, to the exhaustive survey on the preparation of amino acids by Dunn and Rockland, which contains almost 900 references. This review, which also deals with criteria of purity of amino acids and methods of purification used in the Los Angeles laboratory, will be indispensable as a work of reference for anybody engaged in the synthesis of amino acids, but can hardly be recommended as entertaining scientific bedtime reading. J. T. Edsall has contributed a very interesting survey on "The Plasma Proteins and Their Fractionation" with special reference to the results of the Harvard team, which made such important contributions to our knowledge in this field. One of the most interesting aspects of this work is, in the reviewer's opinion, the demonstration that the interaction between proteins and certain low-molecular substances is so very specific. This, and the reactions occurring between different protein molecules and the nature of the forces concerned in these interactions, will undoubtedly become one of the most fruitful fields of protein chemistry. Another very authoritative article is that by R. M. Herriott on the "Reactions of Native Proteins with Chemical Reagents." The main aim of most of the efforts in this field has been to bring about a reaction between a protein molecule and a low-molecular substance under conditions which would produce only a minimum amount of disruption or denaturation. In many cases denaturation could not be definitely excluded, and it must therefore remain doubtful how far some of these reactions are those of the native or of the denatured protein molecule. The field covered by Herriott is, in the reviewer's opinion, only just beginning to develop, and is likely to make very important contributions to our knowledge of protein structure.

A particularly concise and stimulating article is that by L. Michaelis on "Ferritin and Apoferritin," which covers chemical, physical and biological aspects of these interesting substances. The article on "Adsorption Analysis of Amino Acid Mixtures"

by A. Tiselius overlaps slightly with an earlier paper by Martin and Synge in Volume II of this journal. One of the main applications of this very valuable and analytical technique will obviously be the separation of peptides from partial hydrolyzates of proteins. The paper by P. L. Kirk gives a useful survey of methods used for the determination of proteins in biological material. The volume also contains two papers by H. B. Bull and A. Rothen on various aspects of protein films. The considerable amount of work which has been done in this field is probably of greater interest to the surface chemist than to those workers primarily interested in protein structure. The reviewer feels that, on the whole, the contribution made by this particular technique to our knowledge of proteins has been rather disappointing.

The emphasis in the first two volumes has been on the structural, or purely chemical, aspects of proteins. The balance has, at least to some extent, been somewhat restored in the last volume. A. E. Braunstein, who has been so prominently associated with the discovery of biological transamination, contributes a detailed, stimulating and occasionally speculative review on the metabolic role of aspartic and glutamic acids. A. A. Albanese, in a paper on The "Amino Acid Requirements of Man," has collected and critically reviewed a large number of data and observations, many of which were obtained by clinicians and thus not readily available to the experimental biochemist. This review makes it clear that we possess at the moment much more accurate and detailed information on the nitrogen metabolism and amino acid requirements of the rat than of man. R. Elman summarizes recent work on "The Use of Protein and Protein Hydrolyzates for Intravenous Alimentation."

Altogether, it may be considered that Volume III of "Advances in Protein Chemistry" gives a series of well-balanced, up-to-date and critical reviews of many of the most important lines of protein chemistry, and the editors and individual contributors may be congratulated on having produced such a useful and stimulating book.

A. NEUBERGER, London, England

**Chemistry of Muscular Contraction.** By A. SZENT-GYÖRGYI, University of Budapest. vi + 156 pp. Academic Press Inc., New York, N. Y., 1947, Price \$4.50.

This highly stimulating book by the distinguished Hungarian physiologist should be appreciated not only because of its content, but also in view of the circumstances which underlay its preparation and the experimental work on which it is based.

Supported by the Macy Foundation, published in the United States from the Cameron Prize Lecture, held at Edinburgh, Scotland, in 1946, and written by the author in Switzerland, this small volume could be mistaken as a symbol of the re-establishment of the internationality of science. This would, alas, be a highly euphemistic interpretation. In fact, these countries gave shelter and extended help to a renowned scientist whose own laboratory and personal existence were overwhelmed by the holocaust in Europe. This is analogous to similar events in times of war and religious persecution in the 17th and 18th century. But the Thirty Years War gives us a more satisfying comparison when we realize that the valuable experimental work of Szent-Györgyi and his faithful group was carried on from 1940 to 1946 at Szeged while his country was in a turmoil, and he himself was for a long time endangered and often in hiding.

The undaunted human spirit in its devotion to scientific problems can overcome not only personal hardships but acute perils and distress. This reminds us that

Johannes Kepler wrote his system of celestial harmonies, and Otto von Guericke, Burgomaster of Magdeburg, invented the first air pump, likewise in a country ravaged by war. The repetition in our time of such endeavors and sacrifices will be highly comforting to all those who are interested in the human side of natural science.

It is difficult to give an adequate review of the contents of this monograph. Not only the above-mentioned conditions, but likewise the temperament of the author, who represents a most outspoken romantic type of scientific talent, and finally the rapid and turbulent development of this new approach opened by the magic wand of this great experimenter himself, often leave the reader puzzled by apparent contradictions and by an intricate mixture of facts and interpretations difficult to disentangle. The truth and fertility of the theories expounded can be judged only by the future. This is especially true for the continuum theory (Part III), where observations on phosphorescence of gelatin phosphors and similar chromoproteids are used for the explanation of biological units, enzyme systems, and whole cells. But important new observations are scattered through all sections of the book. This is the most unorthodox treatise ever written about muscular contraction. Only in one among seventy recorded papers from Szent-Györgyi's laboratory is the living muscle the direct subject of the investigation. Instead as the author states in the introduction "Like most children, the biochemist, when he finds a toy, usually pulls it to pieces, and he can seldom keep his promise to put it together again. The loveliest toy ever provided by nature for the biochemist is the contractile muscle fibril. . . . When my laboratory started work on muscle some seven years ago, our first step was to pull 'myosin' to pieces."

Indeed, here we encounter one of the most remarkable discoveries described in the book. The classical myosin of Fürth and Edsall is an addition product of two proteins, myosin and actin, which dissociate in the presence of ATP in a special range of KCl concentration. The actin-free (or actin-poor) myosin can be crystallized. The myosin threads of H. H. Weber that actually consist of actomyosin can contract (or superprecipitate) and relax (or dissociate) in the presence of well defined concentrations of KCl and bivalent ions (Mg, Ca) with the addition of physiological doses (or in some cases mere traces) of ATP.

That we are really dealing with the system responsible for the contraction of living muscle may be gathered from the following experiment: "If the muscle is washed in distilled water for several days at 0°, then frozen on the freezing-microtome, cut into slices parallel to the fibers, thawed and suspended in KCl, it contracts violently if ATP is added, similarly to threads of actomyosin. This reaction is very specific and no other substance will elicit such contraction except ATP, the normal constituent of muscle, or substances closely related to it." Here we are not concerned with just any kind of excitation but with the fundamental physicochemical mechanism of contraction itself. The importance of this observation should be duly acknowledged.

The first section describes the properties of myosin, actin, and actomyosin. Whether or not the same concentrations of ATP start either superprecipitation or dissolution of myosin threads depends on the concentration of KCl, the state of myosin, the actin content, the presence of bivalent ions, *etc.*

In the second part (Conditions or Reactions in Muscle) and in the related portion of the fourth section (Recent Advances) the author admits that even now it is impossible to say what the actual state of the system: myosin + actin + ATP + K<sup>+</sup> +



Mg<sup>++</sup> is in the muscle at rest and while active. Only one fact seems rather certain: that rigor of muscle is caused by the formation of insoluble actomyosin when the ATP is exhausted by irreversible dephosphorylation.

In Part IV some of the statements of the foregoing sections are qualified. The ATP-ase activity, according to Szent-Györgyi, is not bound to the pure myosin, but results from the interplay of several components. The rather puzzling statement that myosin in its contracted state only will split ATP seems to be contradicted by the later experiments on ATP-ase activity of muscle "at 0°C. where there is no contraction." Such contradictions clearly result from rapid progress, but they may caution us not to accept as final many of the theories and interpretations. In Part IV the importance of heat- and acid-stable enzyme proteins (these are called protins) is stressed.

Perhaps the most valuable section, especially for the research worker in the field, is Part V (Methods). Here are described the new procedures which enabled the Hungarian workers to attain their remarkable achievements.

This work is surely of the greatest importance for all physiologists and biochemists, especially those interested in muscle. Some of the roughness may perhaps be eliminated in a second edition by incorporating the section "Recent Advances" into the main context.

Undoubtedly the new leads given by Szent-Györgyi will be pursued in many laboratories in America and Europe. Such a response is the most favorable appraisal which can be given to a piece of scientific work.

OTTO MEYERHOF, Philadelphia, Pa.

# The Nutrition of Rainbow Trout. I. Studies of Vitamin Requirements\*

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Received June 4, 1947

## INTRODUCTION

Heretofore, the study of the role of vitamins in fish nutrition has consisted largely of trial and error experiments in connection with epidemics. A number of workers have produced a thiamine deficiency by feeding fresh or frozen fish (1, 2, 3). When fish were fed diets from which fresh meats were excluded, they developed an anemia which was cured by injection of xanthopterin according to Simmons and Norris (4) or by feeding a mixture of riboflavin, pyridoxine and pantothenic acid according to Tunison *et al.* (5).

The latter authors found that the vitamin content of their natural diets could be altered considerably by varying the proportion of the different foods in the ration. Then, by measuring the maximum storage of the respective vitamins in the liver, they believed it possible to determine the tentative requirement. Using this technique, the requirement for riboflavin was found to be 0.6–0.9 mg./100 g. of rations; of pyridoxine 0.4–0.5 mg.; and of pantothenic acid 1.6–1.9 mg.

Since a satisfactory purified ration which permits the growth and well-being of trout has been devised (6) it now becomes possible to determine quantitatively the vitamin requirements of fingerling trout.

## EXPERIMENTAL

All experiments were conducted in the biological laboratory of the James Nevin State Hatchery, Madison. Healthy 4-month old rainbow trout (*Salmo gairdnerii*

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\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

TABLE I  
*Effects of Feeding Vitamin-Deficient Diets on Fingering Rainbow Trout*

Diet no.	Vitamin lacking	Weekly gain				Mortality	Hemo- globin	Liver wt. $\times 100$ body wt.	Liver analysis			Gross symptoms
		First 16 wks.		Next 8 wks.					H <sub>2</sub> O	Fat		
		g.	g.	g.	g.					Per cent	Per cent	
42-3	Control Thiamine	0.89	1.05	1.2	0	9.5	1.2	78.3	2.0	Loss of equilibrium, ano- rexia and pale livers		
42-3-1		0.91	0.85	0.50	53	6.6	0.7	62.3	5.4			
				3.4*	0	10.0	1.1	79.4	2.1			
42-3-2	Riboflavin	0.74	0.68	0.1	40	5.7	0.9	74.3	3.5	Hemorrhagic eyes, nose and operculum		
				2.6*	0	8.3	1.0	75.0	3.3			
42-3-3	Pyridoxine	0.82	0.68	0.4	40	6.4	0.6	76.3	3.2	Nervous disorders, light spots on the liver		
				3.0*	0	10.0	0.9	78.6	2.4			
42-3-4	Pantothenic acid	0.32 (8 wks)	0.1 1.1*	— —	60 0	5.7 9.5	1.1 1.1	78.5 74.4	4.0 3.2	Clubbed gills		
42-3-5	Nicotinic acid	0.69	0.56	0.0	40	5.2	0.9	75.8	3.1	Swollen gills but not clubbed		
				2.8*	0	10.1	1.1	78.0	2.3			
42-3-6	p-Aminoben- zoic acid	0.71	0.65	0.0	40	6.9	0.8	67.2	6.1	Light colored livers		
				1.7*	0	9.5	0.9	76.1	3.8			
42-3-7	Inositol	0.96	0.86	0.3	30	6.0	1.0	79.0	3.3	Degeneration of fin; dis- tended stomachs		
				1.5*	0	8.5	1.1	78.6	2.2			
42-3-8	Choline	0.41	0.1 1.0*	— —	26 0	5.7 7.5	1.6 1.6	76.7 79.0	3.0 2.1	Hemorrhagic kidney and intestine		

TABLE I (Continued)

Diet no.	Vitamins lacking	Weekly gain			Mortality	Hemo- globin	Liver wt. body wt. $\times 100$	Liver analysis			Gross symptoms
		First 16 wks.	Next 8 wks.	Last 4 wks.				H <sub>2</sub> O	Per cent	Fat	
42-3-9	0% Cod liver oil	0.65	0.65	0.4 <sup>a</sup> 1.2 <sup>b</sup> 1.7 <sup>c</sup>	29 29 14	7.2 8.5 8.0	0.9 0.9 1.1	78.2 77.0 77.5	2.5 3.0 2.2		Neurotic kidney
42-3-10	½% Cod liver oil	0.49	0.46	0.0 <sup>d</sup> 1.4 <sup>e</sup> 1.0 <sup>f</sup>	14 56 14	6.6 8.5 9.5	0.9 1.0 1.0	79.5 82.0 79.0	2.5 2.3 3.4		
42-3-11	1% Cod liver oil	0.85	—	—	10	9.7	1.3	78.2	2.6		
42-3-12	Ascorbic acid	0.67	0.0 0.5*	— —	40 0	6.0 7.7	1.2 1.7	76.3 75.9	1.5 2.3		Hemorrhagic liver, kidney and intestine
42-3-13	Alcohol ex- tracted crab meal	0.68	1.0 2.1 <sup>g</sup>	— —	30 0	5.3 8.0	1.4 1.3	78.2 77.8	1.8 2.3		

\* The missing vitamin added to the ration:

<sup>a</sup> Control.<sup>b</sup> D<sub>2</sub> (Ergosterol).<sup>c</sup> D<sub>2</sub> (7-dehydrocholesterol).<sup>d</sup> Control.<sup>e</sup> β-Carotene.<sup>f</sup> Mixture of A<sub>1</sub> and A<sub>2</sub> from fresh water fish liver.<sup>g</sup> Alcoholic extract added to ration.

*irideus*), weighing approximately 3.5 g., were obtained from the rearing tanks where they had received a diet of fresh liver. They were divided into groups of 30, and were treated according to the methods described previously (7). Diet 42-3 which was used as a basal ration in the first series consisted of casein, 52; dextrin, 18;  $\text{CaCO}_3$ , 1; salts IV (8), 6; crab meal, 8; corn oil, 13; cod liver oil, 2; thiamine, 0.2 mg.; riboflavin, 0.3 mg.; nicotinic acid, 10 mg.; *p*-aminobenzoic acid, 60 mg.; choline, 0.4 g.; pyridoxine, 0.2 mg.; inositol, 0.2 g.; pantothenic acid, 0.4 mg.; and ascorbic acid, 0.1 g. Gelatin (10%) was added to the ration to act as a binder and to replace the gelatin capsule employed in the earlier studies.

The modifications used for the determination of the different vitamin requirements are listed in Table I. It will be observed that Diet (42-3-13) differs from Diet 42-3 in that alcohol-extracted crab meal was substituted for the crude product.

Since some of the "supposedly" deficient diets permitted better growth than the controls, it was evident that there must be appreciable quantities of the vitamins present—presumably in the crab meal. It was also shown by chemical analysis of the crude and alcohol-extracted crab meal that substantial amounts of the B complex were removed by alcohol treatment. Further purification of the alcohol-extracted crab meal consisted of digestion in 50% sulfuric acid. The residue (30% yield), thoroughly washed and dried, was incorporated into the vitamin-deficient rations at a level equivalent to its concentration in the crude product. When deficiency symptoms (decreased growth and loss of appetite) became evident, the trout were divided equally into two groups, one of which continued to receive the deficient diet and the other the same diet plus the missing vitamin.

Since a pantothenic acid deficiency developed, using the crude crab meal, 100 additional fish (average weight 5.5 g.) were fed the pantothenic acid-deficient diet. When growth ceased, they were divided into 4 equal groups, one continued on the deficient diet, and the other 3 groups on three different levels of pantothenic acid 0.5, 1 and 2 mg./100 g. of ration. The failure of these levels to cure the gill disease and to produce good growth necessitated a repetition of this experiment with higher levels of the vitamin.

Using Diet 42-3-B, another series was planned to determine the level of thiamine, riboflavin, pyridoxine, choline, inositol, *p*-aminobenzoic acid, pantothenic acid, nicotinic acid, biotin, folic acid, and ascorbic acid required by trout. Twelve gram fish were used in this experiment; 30 fish were used in the control group and 20 in each of the groups on the different levels of the vitamin.

Hemoglobin determinations were made at monthly intervals in all series. When the experiments terminated, the fish were killed and the livers removed and analyzed for fat and moisture content.

## RESULTS

The effect of omitting individual vitamins from the diet on growth, on hemoglobin level, on the size of the liver, and on the moisture and fat content of the liver, as well as the symptoms produced, are presented in Table I.

The effect of supplementing Diet 42-3 with biotin, folic acid, and dried and fresh liver is brought out clearly in Table II.

TABLE II  
*Effect of Supplements in Purified Ration (42-3) on Fingerling Rainbow Trout*  
(30 fish per group)

Diet no.	Supplement	Level of supplement per 100 g. ration	Weekly gain 16 weeks	Hemo-globin	Liver wt. body wt. $\times 100$	Liver analysis	
						Moisture	Fat
			g.	g/100 ml. blood		Per cent	Per cent
42-3	None	—	0.89	9.5	1.2	78.3	2.0
42-3-A	Biotin	5 $\gamma$	0.82	8.2	2.0	78.2	2.2
42-3-B	Biotin	5 $\gamma$					
	Folic acid	10 $\gamma$	1.04	9.5	1.0	80.2	2.4
42-3-C	Dried liver	2 g.	0.92	8.5	1.3	79.1	2.1
42-3	Fresh liver	20 g.	0.80	9.5	1.1	78.2	2.4

The results of the last series, in which the fish were fed Diet 42-3-B with different levels of the 11 vitamins tested previously, are summarized in Table III.

The optimum range of each of the vitamins studied was found to be as follows:

	mg./100 g. ration
Thiamine	between 0.1 and 1
Riboflavin	between 0.5 and 1.5
Pyridoxine	between 0.1 and 1.0
Pantothenic acid	between 1.0 and 2.0
Nicotinic acid	between 0.1 and 0.5
Choline	between 5.0 and 10.0
Inositol	between 25.0 and 50.0
p-Aminobenzoic acid	between 10.0 and 20.0
Biotin	between 0.005 and 0.025
Folic acid	between 0.1 and 0.5
Ascorbic acid	between 25.0 and 50.0

## DISCUSSION

The results of these experiments indicate that growth is significantly affected by a lack of the following vitamins: thiamine, inositol, pantothenic acid, nicotinic acid, and ascorbic acid. In contrast to the rat and chicken, which are extensively used in nutrition experiments, trout

show a definite maximum in growth rate with certain levels of the vitamins. This is clearly shown in all the vitamins studied. The depressing effect of higher levels of the vitamins on growth increases the difficulty of ascertaining the true requirement of the vitamin. Trout are the only animals in which it has been possible to demonstrate a

TABLE III

*Effect of Feeding Various Levels of Vitamins in Purified Ration on Fingerling Rainbow Trout*

Vitamin under test	Level mg./ 100 g. ration	Weekly gain	Hemoglobin	Liver wt. body wt. $\times 100$	Liver analysis	
					Moisture	Fat
		<i>g.</i>	<i>g./100 ml. blood</i>		<i>Per cent</i>	<i>Per cent</i>
Thiamine	0	0.29	9.5	0.9	67.8	3.1
	0.1	0.73	10.0	1.2	76.0	1.3
	1.0	0.79	10.0	1.2	76.0	1.8
	2.0	0.75	10.1	1.3	78.9	2.3
Riboflavin	0	0.70	9.3	1.1	78.8	1.0
	0.5	1.40	9.0	1.2	78.9	1.7
	1.5	1.50	12.0	1.2	78.5	2.2
	3.0	0.75	10.1	1.3	78.9	2.3
Pyridoxine	0	0.76	9.5	1.3	71.0	2.0
	0.1	1.40	10.0	1.1	75.8	2.7
	1.0	1.30	9.0	1.1	77.0	2.3
	2.0	0.75	10.1	1.3	78.9	2.3
Choline	0	0.54	10.0	1.1	79.3	1.2
	5.0	0.71	10.8	1.1	77.6	1.6
	10.0	0.81	9.0	1.1	78.3	2.4
	400.0	0.75	10.1	1.3	78.9	2.3
Ascorbic acid	0	0.19	9.0	1.1	78.6	1.5
	25.0	1.00	11.0	1.3	78.5	2.5
	50.0	0.75	9.0	1.3	78.7	2.6
	100.0	0.75	10.1	1.3	78.9	2.3
Inositol	0	0.34	8.3	0.7	77.3	3.0
	25.0	1.00	8.9	0.9	68.8	2.5
	50.0	1.10	9.8	1.1	77.3	2.8
	200.0	0.75	10.1	1.3	78.9	2.3

TABLE III (Continued)

Vitamin under test	Level mg./ 100 g. ration	Weekly gain	Hemoglobin	Liver wt. body wt. $\times 100$	Liver analysis	
					Moisture	Fat
		<i>g.</i>	<i>g./100 ml. blood</i>		<i>Per cent</i>	<i>Per cent</i>
<i>p</i> -Aminobenzoic acid	0	0.70	8.5	1.8	75.7	3.7
	10.0	1.30	10.5	1.4	77.9	2.0
	20.0	1.10	9.0	1.2	78.4	2.1
	60.0	0.75	10.1	1.3	78.9	2.3
Pantothenic acid	0	0.73	9.0	1.5	78.1	3.5
	1.0	1.20	10.0	1.0	70.1	3.0
	2.0	1.60	11.0	1.5	78.7	2.4
	4.0	0.75	10.1	1.3	78.9	2.3
	6.0	0.80	9.5	1.0	76.7	2.1
Nicotinic acid	0	0.28	8.0	1.3	75.7	3.1
	0.1	1.00	9.5	1.0	78.0	2.3
	0.5	0.80	8.3	1.1	78.7	2.2
	1.0	0.61	8.7	1.2	78.6	2.4
	10.0	0.56	8.5	1.2	77.4	2.2
Biotin	0	0.63	9.3	1.8	79.6	1.5
	0.005	0.85	9.2	1.7	80.0	1.2
	0.025	0.80	9.8	1.4	79.7	1.4
	0.100	0.70	9.5	1.6	77.8	2.7
Folic acid	0	0.61	8.0	2.3	80.5	1.8
	0.010	0.85	9.2	1.7	80.0	1.2
	0.100	1.2	9.6	1.8	78.2	1.2
	0.500	1.0	11.0	1.7	76.2	1.5

growth stimulation by *p*-aminobenzoic acid in the presence of folic acid. Possibly this is due to a greater need for folic acid than Diet 42-3-B supplies.

The growth rate depression due to high biotin was counteracted by folic acid. However, when these two vitamins were added, the best weight gain of the series was obtained. The addition of either 2% dried liver or 20% fresh liver retarded the growth rate.

Reports by the New York workers (5, 9) that an anemia is produced when riboflavin, pyridoxine, and pantothenic acid are omitted from the diet were substantiated by the results of Series I, in which the vitamin



deficiencies were produced in the absence of biotin and folic acid. However, in the second series, when Diet 42-3-B containing biotin and folic acid was used, no anemia developed. It is apparent, then, that folic acid and/or biotin is specific in the prevention of anemia, and that the anemia produced by the omission of the other vitamins was probably due to an indirect effect.

Liver pathology, similar to that produced in earlier experiments, was not observed in any of the fish. In the first series, a slight reduction of size was observed and could possibly be attributed to anorexia which is known to have such an effect.

The chemical analysis of the livers brought to light two interesting facts, namely, that the fat content of thiamine and *p*-aminobenzoic acid-deficient groups was more than double that of the controls and also twice that reported for wild trout (10). In all the vitamin-deficient groups the fat content of the liver tended to differ from that of the controls.

The gross symptoms observed in the thiamine-deficient fish were very similar to those reported by various investigators who fed fresh or frozen fish, containing thiaminase (11). The exclusion of riboflavin from the diet caused no consistent symptoms of disease. About 40% of those dying, however, during the course of the experiment, did have hemorrhagic eyes, nares, and operculum. Trout, like many other animals, develop nervous disorders when pyridoxine is omitted from the diet. They appear to lose their ability to judge distances, a lack of which is noticeable in their futile attempts to capture pieces of food dropping down into the water.

The large proportion in this ration of casein, which has a high methionine content, probably prevents the excessive deposition of fat in the liver, when trout are maintained on a choline-deficient diet. Kidney degeneration and hemorrhagic intestines were observed, however, in the absence of the vitamin.

The trout fed the inositol-deficient diet developed a fin degeneration which is very similar to fin rot (13). The fin, when examined microscopically, appeared to be disintegrating. Hexachlorobenzene 666, a compound similar in structure to inositol, when added to the diet for 4-6 days, caused a similar degeneration. The normal appearance of the fins was restored by withholding the drug for 3-5 days.

The omission of pantothenic acid caused an early retardation of growth followed by the development of club-shaped gills which, ac-

cording to Wolf (13) and Tunison *et al.* (9), is similar to the Western type of Gill Disease. The feeding of 4 mg. of pantothenic acid/100 g. ration alleviates the condition in 4-6 days.

Swollen gills were observed in all trout on a nicotinic acid-deficient diet. The severity of the condition is indicated by the fact that at the time of death the position of the operculum in relation to the body was almost at a right angle.

The vitamin C-deficiency produced in fingerling trout seemed to be irreversible. The hemorrhagic condition of the intestine, liver and kidney was not significantly improved when ascorbic acid was restored to the diet.

During the 7 months on experiment, the fish on the cod liver oil-free diet, Diet 42-3-9, grew very slowly, but continuously, and exhibited no apparent abnormality except a kidney degeneration. Due to some unknown cause the diet containing 0.5% cod liver, Diet 42-3-10, when fed, produced poorer growth in the fish than the cod liver oil-free diet. Diet 42-3-11 (1% cod liver oil) produced almost as good growth as the basal ration.

Vitamin D<sub>3</sub> (irradiated 7-dehydrocholesterol) appeared to be more effective than vitamin D<sub>2</sub> (irradiated ergosterol) as a source of vitamin D.

The crude mixture of vitamin A<sub>1</sub> and A<sub>2</sub> prepared from fresh-water fish livers and containing approximately the same amount of vitamin A as 2% cod liver oil gave a moderate growth response. On the other hand,  $\beta$ -carotene exhibited a toxic effect.

#### ACKNOWLEDGMENTS

We are indebted to Merck and Co., Inc., Rahway, N. J., for the generous supply of crystalline B vitamins, and to Seaboard Supply Company, Philadelphia, Pa., for crab meal.

We also wish to acknowledge the various vitamin determinations carried out on the crab meal, both crude and alcohol-extracted, by Virgil Wiebelhaus, John B. Neilands, A. L. Powell and Andrew Halverson.

#### SUMMARY

1. By using a purified diet in which individual vitamins were omitted, indications of deficiency, such as decreased growth rate and symptoms of disease, were produced in trout.

2. The optimum levels of thiamine, riboflavin, pyridoxine, panto-

thenic acid, nicotinic acid, choline, inositol, *p*-aminobenzoic acid, biotin, folic acid, and ascorbic acid with this diet have been determined.

3. The relation of the vitamins to specific disease symptoms has been discussed.

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## The Nutrition of Rainbow Trout. II. Further Studies with Purified Rations\*

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Received June 4, 1947

### INTRODUCTION

Experiments in a preceding publication (1) showed that yearling rainbow trout fed a purified ration (Diet 18) consisting of cerelose, 20; casein, 68; corn oil, 2; with dried liver and yeast, maintained hemoglobin levels and growth rates equal to those produced by feeding a standard hatchery ration (fresh meat). Large scale nutrition experiments with practical diets indicated that fingerlings, as might be expected, were more susceptible than yearlings to dietary deficiencies. Therefore, it was thought advisable to use the younger, faster-growing fish for studies with purified rations. The purpose of this paper is to present evidence that a purified diet can meet the nutritional requirements of fingerling trout.

### EXPERIMENTAL

All experiments were conducted in the biological laboratory of the James Nevin State Hatchery, Madison. Healthy seven month old trout (*Salmo gairdnerii irideus*) were obtained directly from the rearing tanks in the hatchery, where they had been fed fresh liver. Fish weighing 12-14 g. were selected and divided into groups of 30. The laboratory techniques were identical with those previously described (1).

The constituents of the basal ration (Diet 27) and other modified rations based on Diet 18, and Diets 41 and 42 based on the data presented by Embody and Gordon (2) concerning the chemical analysis of stomach contents of wild trout are listed in Table I.

Since Diet 41 gave the best results, a second series was designed in order to more completely purify the diet, and to establish the optimum salt level. The modifications of Diet 41 are listed in Table II.

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\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

TABLE I  
Composition of Diets used in Preliminary Studies with Results

Constituents	27	28	29-1	29-2	29-3	30-1	30-2	30-3	31-1	31-2	31-3	32	33-1	33-2	34	35	36	37	38	40	41	42	Stock
	Percentages																						
Casain	65	65	64	63	61 <sup>c</sup>	64	63	61	70	73	75	45	45	25	61	61	57	55	65	65	42	42	
Dextrin	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	18	18	
Brewers' yeast	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	
Dried liver	5	5	5	5	5	5	5	5	5	2	5	5	5	5	5	5	5	5	5	5	5	5	
Corn oil	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	13	3	3	13	13	
Crab meal																							
Cellulflour																							
Kelp												20	40				4						
B-G plus																	4						
Lyophilized liver																	4						
Whole liver substance																			5				
Salts (IV) (3)																				5	6	6	
CaCO <sub>3</sub>																					1	1	
Vitamin mixture <sup>a</sup>																						+	
Weekly gain (16 wks.) (g.)	2.1	2.1	2.2	1.4	2.1	2.2	2.1	2.3	2.2	2.2	1.5	1.8	2.0	1.3	2.3	2.2	2.2	2.6	1.8	2.2	3.1	2.7	1.0
Hemoglobin (g./100 ml. blood)	8.0	8.9	7.6	7.7	8.4	10.4	6.5	7.9	8.2	7.2	5.6	7.2	6.7	9.7	9.1	8.6	7.8	9.0	7.5	8.0	8.9	8.5	9.3
Mortality (No. of fish)	1	1	1	15	0	0	0	0	1	13	15	1	0	0	2	1	5	0	2	2	2	1	0
Liver wt. $\times 100$	3.6	3.0	3.3	4.3	3.5	4.2	3.6	3.2	4.1	3.7	4.2	2.9	3.1	2.5	3.4	3.4	2.8	3.2	3.6	2.9	2.1	2.8	1.5
Body wt.	Y	R	Y	Y	Y	R	R	R	R	R	R	R	Y	Y	Y	Y	Y	R	Y	Y	R	R	
Liver color <sup>b</sup>																							

<sup>a</sup> The vitamin mixture consisted of thiamine hydrochloride 20 mg., riboflavin 30 mg., pyridoxine 20 mg., calcium pantothenate 40 mg., niacin 100 mg., *p*-aminobenzoic acid 600 mg., choline chloride 4 g., and *i*-inositol 2 g./kg.

<sup>b</sup> Y = Yellow. R = Red.

Vitamin C was added 0.1 g./100 g. of ration. 2% Cod liver oil was used as a source of vitamins A and D.

TABLE II  
Composition of Basal Diet 41 and Its Modifications with Results

Constituents	Percentages																	Stock
	41	42	41-1	42-1	41-2	42-2	41-3	42-3	42-4	42-5	42-6	42-7	42-8	46	47-1	47-2	48	
Casein	42	42	47	47	47	47	52	52	50	42	46	44	40	37	42	42	42	
Fibrin	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	
Dextrin	6	6	6	6	6	6	6	6	6	6	2	4	8	6	6	6	6	
Salts (IV)	1	1	1	1	1	1	1	1	1	1	2	4	8	1	1	1	1	
CaCO <sub>3</sub>	13	13	13	13	13	13	13	13	13	13	13	13	13	18	13	13	13	
Corn oil	2	2	2	2	2	2	2	2	2	2	8	8	8	2	2	2	2	
Cod liver oil	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	
Crab meal	5	5	5	5	5	5	5	5	2	10	5	5	5	5	5	5	5	
Cellulose	5	5	5	5	5	5	5	5	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	
Dried liver	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	+	+	+	+	+	+	+	+	+	
Brewers' yeast	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Ascorbic acid																		
Vitamin mixture																		
Weekly gain (16 wks.) (g.)	1.9	1.8	2.1	1.8	2.2	2.2	1.3*	2.7	2.5	1.1*	3.0	1.7	2.2	1.2	2.0	1.6	2.2	
Hemoglobin (g./100 ml. blood)	12.5	11.4	8.1	8.2	7.9	7.4	5.6	8.4	8.0	8.8	8.9	7.6	8.0	7.2	8.6	10.0	9.0	
Mortality (No. of fish)	0	1	18	2	3	1	20	0	3	0	2	4	2	1	9	0	2	
Body wt. X 100	1.7	1.5	2.0	1.4	2.0	1.6	2.3	2.0	1.8	1.8	1.7	1.5	1.8	1.4	1.4	1.7	2.0	
Liver analysis	83.2	87.5	89.0	82.2	82.8	82.8	72.6	74.5	75.0	76.3	76.8	80.8	78.0	80.5	79.2	77.8	82.8	
Moisture	4.1	2.5	2.4	2.0	2.4	4.9	4.8	4.1	3.4	1.9	3.1	2.1	4.2	1.9	2.0	2.3	2.2	
Fat																	1.1	

\* 10 wk. group divided, one continued on same diet, other group supplemented with vitamin mixture.

† 10 wk. group divided, one continued same diet, other group given same diet with corn oil substituted for liver fat.

While Diet 42-3, free from natural sources of accessory factors such as liver or yeast, and containing crystalline vitamins, did not produce optimal growth, it proved to be the most promising for further studies. By varying the amounts of fat (3, 8, 13 and 18%) this diet served to determine the optimal level of that constituent for trout.

Because of the inconsistencies occurring with the salt levels outlined in Tables I and II, the favorable growth responses obtained earlier with fibrin and CellufLOUR as substitutes for casein and crab meal respectively, Diet 51, in which these substances were incorporated, was devised for further testing of the salt levels. The basal diet for this series consisted of: fibrin, 52; dextrin, 18; CellufLOUR, 8;  $\text{CaCO}_3$ , 1; corn oil, 13; cod liver oil, 2; ascorbic acid, 0.1 g. and the crystalline vitamin mixture. Salt levels of 2, 4, 6 and 8% were used and were compensated for by varying the fibrin. Only four-month old fingerlings (average weight 3.5 g.) were available for this experiment, hence capsule feeding had to be discarded and the fish were fed *ad libitum*. To maintain the proportion of the main food constituents previously fed in capsules, and to provide a binder for the ration, 10% finely ground gelatin was added. The mixture was moistened with water, permitted to coagulate, and fed as particles.

To determine the constituent, or constituents, of crab meal which was responsible for the growth stimulation, the following fractions were incorporated in Diet 42-3 in amounts equivalent to the original 8% level:

- (a) Alcohol-extracted crab meal—48 hour extraction with 95% ethyl alcohol.
- (b) Alcoholic extract of crab meal.
- (c) Ether-soluble fraction of alcoholic extract.
- (d) Water-soluble fraction of alcoholic extract.
- (e) Acid-treated crab meal—demineralized with 10% trichloroacetic acid, washed to remove calcium salts, then hydrolyzed with 50% sulfuric acid, washed, and dried. Yield 30%.
- (f) Ash—heated gradually to 1200°C. and maintained at that temperature for 48 hours. Yield 26%.

With each series a control groups of fish was maintained which received fresh liver to the extent of approximately 6% of their body weight per day. The criteria used for judging the adequacies of the diet were growth response, size and color of the liver and the hemoglobin level of the blood. Representative fish were autopsied during the course of the experiment in order to make observations on the internal organs. The livers were removed, weighed, and analyzed for fat and moisture content.

## RESULTS AND DISCUSSION

From Table I, which gives a summary of the studies in Series I, it is apparent that Diet 41, containing crab meal and 13% fat, more nearly satisfied the nutritional requirements than any of the other rations tested. Furthermore, the fish on this diet, those on Diet 32 containing crab meal, and those on Diet 37 containing 13% fat, were the only animals which maintained normal colored livers throughout the 16-week experimental period, even though the vitamin mixture was absent from the rations.

Using Diet 41 as a basal ration in the second series, it was possible to eliminate both liver and yeast as accessory foods by using the vitamin mixture as in Diet 42-3 (Table II).

Removal of both liver and yeast in the absence of the vitamins (Diet 41-3) caused poor growth, anemia, enlarged livers and a high mortality rate. At the end of 10 weeks, the surviving fish in this group were divided into two groups, one continued on the same diet and the other given Diet 42-3, which contained the vitamin supplement. In the supplemented group, growth rate and hemoglobin values were dramatically improved and the mortality rate decreased. The addition of 2% dried liver to Diet 42-3 gave no marked improvement while 10% definitely retarded the growth. Growth could be restored, however, if the fish were fed a diet similar in all respects except that ether-extracted dried liver plus an amount of corn oil equivalent to the liver fat extracted was substituted. The fat extracted from the dried liver contained large amounts of vitamin A, which was presumed to be the factor responsible for poor growth.

If one compares the results in Tables I and II it is evident that values for the rate of growth, for the hemoglobin content of the blood, *etc.*, for fish given similar diets show considerable variation. This is to be expected since different lots of fish were used, the series were started at different periods of the year and some of the supplements were from

TABLE III  
*Effect of Feeding Purified Rations with Different Levels of Fat  
and Salts on Fingerling Rainbow Trout*

Diet no.	Variable	Weekly gain 16 weeks	Hemoglobin	Mortality	Liver wt. body wt. $\times 100$	Liver analysis	
						Moisture	Fat
	Corn oil Per cent	g.	g./100 ml. blood	No. of fish		Per cent	Per cent
42-3	13	0.89	8.7	0	1.1	78.2	2.5
42-3F <sub>1</sub>	3	0.92	8.4	3	2.1	77.8	0.50
42-3F <sub>2</sub>	8	0.87	8.7	2	1.8	79.2	1.5
42-3F <sub>3</sub>	18	0.72	8.1	3	1.5	80.1	1.5
	Salts IV Per cent						
51-1	2	0.38	8.0	3	1.9	77.9	2.2
51-2	4	0.44	7.5	2	2.3	81.9	1.7
51-	6	0.73	7.8	0	1.7	79.9	2.3
51-3	8	0.43	8.9	3	—	—	—



different source materials. This variation is not serious since proper control groups were included in each series.

The results obtained by feeding different levels of corn oil are presented in Table III. It is apparent that levels of 3, 8 and 13% gave almost identical results if only growth rate and hemoglobin levels are considered. If mortality rate and liver fat are also used, then it appears that the 13% level was optimal.

Early attempts in Series I and II to determine the optimum salt level failed to yield any conclusive evidence. However, when Diet 51, the constituents of which were selected for their low salt content, was used, a definite relationship was shown between the salt content and growth response. The results presented in Table III indicate the decided advantage of the 6% salt level.

Having developed a purified ration that promoted growth and well-being of fingerling rainbow trout, an attempt was made to determine roughly the nature of the growth-promoting qualities of crab meal. In the course of these experiments, it became evident that the crude crab meal contained appreciable amounts of the B vitamins. To determine whether these vitamins could be removed, the crab meal was fractionated and the various fractions incorporated in Diet 42-3 at levels equivalent to the original 8% level.

It is obvious from the results presented in Table IV that the most completely purified fraction from crab meal, sulfuric acid treated, gave

TABLE IV  
*Effect of Feeding Crab Meal or its Fractions in a Purified  
Ration to Fingerling Rainbow Trout*

Diet no.	Crab meal treatment	Supplement	Weekly gain 10 weeks	Hemo-globin	Mortality	Liver wt. body wt. X100	Liver analysis	
							Moisture	Fat
			g.	g./100 ml. blood	No. of fish		Per cent	Per cent
42-3 1A	None	None	0.56	10.2	2	1.1	78.3	2.0
42-3 2A	Alcohol extracted	None	0.49	9.2	2	1.3	77.8	1.0
42-3 3A	Alcohol extracted	Alcoholic extract	0.49	10.5	0	1.2	75.6	3.7
42-3 4A	Alcohol extracted	Water soluble of alcoholic extract	0.48	9.0	1	1.3	75.7	2.4
42-3 5A	Alcohol extracted	Ether solubles of alcoholic extract	0.58	10.0	1	1.3	78.2	1.6
42-3 6A	Alcohol extracted	Alcoholic extract	0.56	7.8	1	1.5	78.2	2.0
42-3 7A	Acid	None	0.64	10.1	0	1.3	78.9	2.3
42-3 10A	Ash	None	0.36	4.9	4	1.5	78.8	2.4
42-3 8A	Crab meal omitted		0.51	8.2	6	1.2	78.6	1.9
42-3 8A	Cellulofur replacing crab meal		0.49	9.6	6	1.2	79.6	1.5

as good growth as the untreated meal. However, the active material was destroyed when the meal was ashed. The ether-soluble fraction of the alcoholic extract may be important in supplying some of the nutritional requirements of trout.

#### ACKNOWLEDGMENTS

The authors are indebted to Merck and Co. Inc., Rahway, New Jersey, for the generous supply of crystalline B vitamins; to Philip R. Park, Inc., San Pedro, Calif., for help and B-G plus; to Wilson Labs., Inc., Chicago, Ill., for the liver preparation; and to Seaboard Supply Co., Philadelphia, Pa., for crab meal.

#### SUMMARY

1. A purified ration containing dextrin, 18; casein, 52; crab meal, 8; salts IV, 6;  $\text{CaCO}_3$ , 1; cod liver oil, 2; corn oil, 13; plus the crystalline B vitamins and ascorbic acid, has been shown to produce good growth in fingerling rainbow trout. No pathology was observed in the fish livers but somewhat lowered hemoglobin values were obtained.

2. The optimum level of salts IV has been shown to be 6% when fibrin and CellufLOUR are substituted for casein and crab meal, respectively.

3. The optimum level of corn oil was found to be 13%.

4. The most important constituent of the crab meal was left in the residue after sulfuric acid treatment.

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# **The Effect of Phthalylsulfathiazole (Sulfathalidine) on the Excretion of B-Vitamins by Normal Adults**

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Received September 26, 1946

## **INTRODUCTION**

The administration of sulfonamides in the presence of purified diets has come into recent use as a means of studying the requirements for certain vitamins (1). Thus, it has been concluded that biotin and "folic acid" are essential for the rat (2, 3, 4, 5, 6) because adverse effects upon growth and well-being produced when sulfonamides are fed can be dispelled by the addition of these nutrients. Other investigators report a decreased fecal excretion of pantothenic acid as well as biotin and "folic acid" when rats are fed succinylsulfathiazole or phthalylsulfathiazole (7). Sulfonamides have been similarly used in human nutrition studies. A sharp drop in fecal free thiamine was reported when succinylsulfathiazole was given in the presence of a controlled intake of purified diets (8). No similar change in the fecal output of riboflavin was found, however (9). A reduction in urinary *N*<sup>1</sup>-methylnicotinamide was reported to be caused by succinylsulfathiazole (10); however contradictory results have been reported in another study (11).

Concurrent with these studies, there have been several investigations with both animals and humans, designed to study the effect of sulfonamides upon the numbers and types of bacteria found in feces (6, 7, 12, 13, 14, 15). It appears from these studies that the greatest

\* An installation under the jurisdiction of the Office of the Surgeon General, U. S. Army.

drop in bacterial numbers occurs with the coliform microflora, the "total count" remaining relatively constant.

B-vitamin excretion by young men receiving normal and restricted intakes of these vitamins has been previously reported by this laboratory (16, 17). The purpose of this report is to present and discuss the urinary and fecal output of the B-vitamins, *L. casei* factor, pyridoxine, pantothenic acid, biotin, thiamine, riboflavin, nicotinic acid and *N*<sup>1</sup>-methylnicotinamide, when these subjects received phthalylsulfathiazole in therapeutic amounts. Associated changes in the bacteriologic counts of the feces were also determined.

## EXPERIMENTAL

### General

The subjects were 5 normal young men aged 23 to 28, judged to be free from significant organic disease or defects. These men consumed carefully measured amounts of an experimental diet that was assayed weekly for its vitamin content. Their activity consisted of attending classes in the university, doing laboratory or clerical work, participating in sports, *e.g.*, basketball, tennis, hikes, *etc.*, according to their personal desires. Further details pertaining to the subjects, their diets, the methods of urine and feces collections, and microbiological and chemical assay procedures employed are given in other publications (16, 17, 18). The only difference in technical procedures was the inclusion of 5 mg. of *p*-aminobenzoic acid per 100 ml. of the microbiological assay media when fecal samples contained sulfonamide in order to counteract any possible interference of the drug on the growth of the assay organisms. This addition was shown subsequently to have no effect upon assay values.

### Diet

The basal diet consumed by all subjects contained restricted amounts of B-complex vitamins, and corn comprised approximately 27% of the caloric intake. In addition, 2 of the subjects received crystalline supplements of thiamine, riboflavin

TABLE I  
*Daily Intake of B-Vitamins*

Vitamin	Subjects 1, 3, and 5	Subjects 6 and 7
Thiamine, mg.	2.1	2.1
Riboflavin, mg.	2.0	2.0
Nicotinic Acid, mg.	20	20
Biotin, $\gamma$	78	18
<i>L. casei</i> factor, $\gamma$ .	111	21
Pantothenic Acid, mg.	7.7	1.7
Pyridoxine, mg.	4.3	1.3

nicotinamide, as well as the lesser-known B-complex factors (*L. casei* factor, pyridoxine, pantothenic acid, biotin, choline), all given in amounts to equal or slightly exceed the levels found in a normal diet. The remaining 3 subjects were on the same regime, but lacked the supplements of the "lesser-known" factors.

The actual intake of nutrients for the two groups of men is shown in Table I.

Phthalsulfathiazole was chosen as the sulfonamide because of its slow absorption from the intestinal tract, its low toxicity, and its marked bacteriostatic activity in the human intestine (19). The drug was administered 4 times daily, 1 g. at each meal and at bed time. The subjects were given the drug for intervals of from 8 to 17 days.

### Bacteriological Analysis

Stool samples for bacteriological counts were collected during the day, refrigerated (not frozen) immediately after voiding and plated the same day. With but few exceptions, samples were less than 12 hours old when plated. Bacteriological examinations were made weekly before the drug was administered, twice a week during the period of sulfonamide administration and on two occasions in the week following its withdrawal.

Sterile physiological saline was employed in making the dilutions for plating. Five to ten grams of feces were homogenized in 100 ml. of sterile saline by means of a Waring Blendor and used for subsequent dilution. The solid content of this suspension was determined by centrifuging an aliquot in a graduated centrifuge tube. All counts were calculated as number of microorganisms per ml. of wet solids.

Eosin-methylene blue agar incubated for 48 hours at 37°C. was used for the coliform plate counts. Bacto blood agar base plus 3% sterile citrated sheep's blood was employed for the "total" aerobic plate count. The aciduric microorganisms, lactobacilli and streptococci were enumerated using Bacto trypsin digest agar plates incubated anaerobically at 37°C. for 72-96 hours. Longer incubation periods gave no increase in numbers of colonies with any of the media. Five mg. of *p*-aminobenzoic acid/100 ml. of media were used in plating samples containing sulfonamide. Media containing no *p*-aminobenzoic acid gave no difference in count in a number of trials, but the material was included as a precaution against inhibitory effects.

Isolations were made throughout the study from the countable plates of all 3 media. These isolations were studied to ascertain which types of microorganisms predominated during the different phases of the experiment. Coliform bacteria were identified by colony morphology, growth in brilliant green bile broth, and the "Invic reactions." Streptococci were identified by their gram morphology. The lactobacilli were identified by their distinctive colony morphology and the irregularity of cell form typical of freshly isolated intestinal lactobacilli. A number of isolations were further studied. Both *Lactobacillus acidophilus* and *Lactobacillus bifidus* types were found.

## RESULTS

### Fecal Excretion

The only marked decrease in fecal excretion of B-vitamins occurred in the levels of *L. casei* factor. There was a sharp drop in excretion

levels coinciding with the intake of sulfonamide, followed by a rapid rise when the drug was withdrawn. (See Fig. 1A). In addition, the excretion of biotin was also affected, but the decrease was much less marked. Biotin excretion values for all 5 subjects decreased significantly during the period of drug feeding and increased when the feeding of the drug was discontinued. (See Fig. 1B.) In spite of the lack of a sharp drop in fecal excretion of biotin, it is believed that the results corroborate previous findings of a decreased biotin output in rats receiving sulfonamides (7).

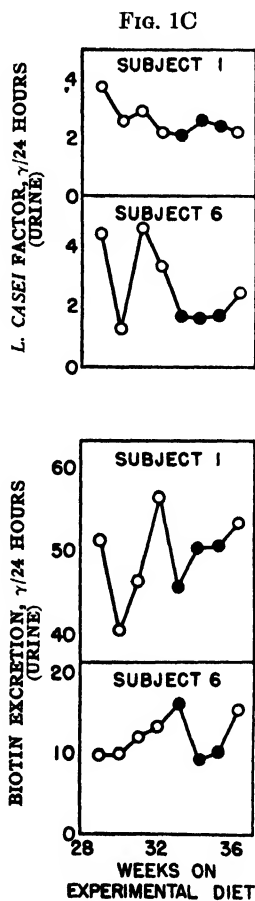
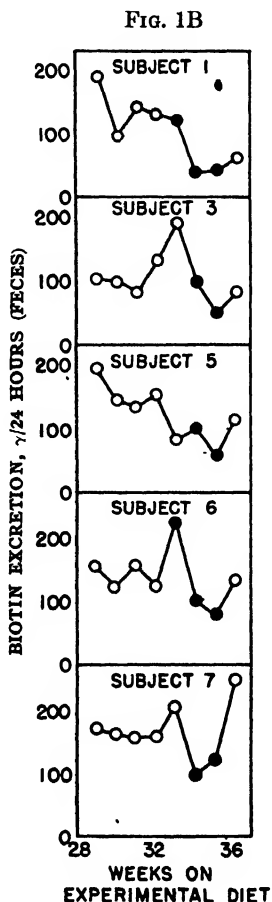
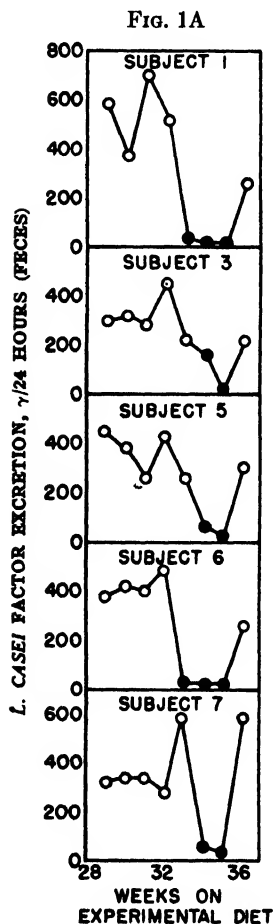


FIG. 1D

FIGS. 1A-1D

There were no conclusive evidences of a generally decreased excretion of any of the other B-vitamins, although such was suggested in certain of the subjects. Subjects 1, 3 and 6, whose coliform counts dropped more sharply than did those of subjects 5 and 7, demonstrated slightly decreased outputs of thiamine, and two of these subjects, 1 and 3, also showed a decrease in excretion of riboflavin and pantothenic acid. These decreases were followed by an increase when the use of the drug was discontinued. There was no evidence of a decrease in fecal pyridoxine and only subject 3 showed evidence of decreased fecal nicotinic acid.

### *Urinary Excretion*

No evidence was found which would indicate a decreased urinary output of any of these B-vitamins, with the possible exception of *L. casei* factor in two of the subjects. Furthermore, the evidence which indicated a drop in urinary excretion levels of these two subjects was not conclusive. The data for *L. casei* factor and biotin, representing those subjects in whom the drop in fecal excretion was greatest are shown in Figs. 1C and 1D. The lack of any decisive drop in urinary *L. casei* factor and biotin output corresponding to that found in the feces can be interpreted in a number of ways. It may be that fecal vitamins do not contribute to the metabolic supply; yet this is unlikely when it is considered that rats require additional *L. casei* factor when fed sulfonamide drugs. However, a species difference may enter to prevent direct comparison between the situation in rats and that occurring in the human. Another possible explanation is that there may have been insufficient time for a decrease in urinary excretion to occur. This could be true, especially if the amount of this vitamin obtained from intestinal synthesis were small, or if considerable quantities of the vitamin were still stored in the liver and other tissues. A third possibility is that the chief metabolite of *L. casei* factor was not measured.

### *Bacterial Counts*

The interesting features of the bacteriological findings were (1) the inhibitory effect of the sulfonamide upon the coliform flora, and (2) the variation among individual subjects as to numbers and types of micro-organisms, this being unrelated to dietary supplementation. Subjects



6 and 7, whose intestinal flora varied markedly from each other, were on identical diets for over six months. Table II presents the changes found in bacterial counts, and also indicates the degree of fluctuation in counts found among individuals. Wide variations in fecal bacterial counts are common (20).

TABLE II

*Bacterial Counts of Feces*

(Number of bacteria, millions/g. wet feces)

Subject no.	Type of organism	Time of stool collection						
		1 day before beginning sulfathalidine	days after beginning of sulfathalidine					8 days after drug withdrawal
			2	6	9	13	17	
1	Coliform	2000	.01	.0001	.004	.005	<.0001	22
	Total aerobic	*	.14	.07	.04	18	.88	20
	Aciduric	*	210	140	65	100	380	690
3	Coliform	0.16	.002	.002	—	—	—	2
	Total aerobic	2.1	1.9	4.9	—	—	—	3.5
	Aciduric	680	740	300	—	—	—	3900
5	Coliform	0.9	1.1	.01	.15	—	—	1.9
	Total aerobic	15	2.1	5.7	4.5	—	—	2.6
	Aciduric	72	25	38	1120	—	—	1070
6	Coliform	20	.04	.0002	.04	<.0001	<.0001	14
	Total aerobic	24	.16	.95	.14	2.4	.34	12
	Aciduric	750	40	33	—	26	160	1500
7	Coliform	180	.005	.16	.27	—	—	2.5
	Total aerobic	*	.7	3.2	5.2	—	—	13.5
	Aciduric	*	2.5	6.5	9.4	—	—	260

\* Coliform bacteria obscured all other types.

The decline in coliform counts, while notable in all 5 subjects, was distinctly greater in the cases of subjects 1, 3 and 6 than in 5 and 7. Aciduric organisms, chiefly lactobacilli, were less affected by the drug, and even increased in the case of subject 5. One effect of the drug was to produce a predominantly aciduric flora in two subjects having a coliform type previously. All 5 subjects demonstrated a predominantly aciduric microflora 8 days after the drug was withdrawn.

The number of streptococci was less affected than that of the coliform organisms. While the number of streptococci upon the blood plates showed no great change, it was observed that the small cell long chain type disappeared when the feces contained the drug and were replaced by the larger cell shorter chain streptococci.

The data indicate a relation between the number of coliform organisms found in the intestinal contents and the bacterial synthesis of *L. casei* factor, and to a lesser extent the synthesis of biotin. This relationship was indefinite or was absent for the other B-vitamins studied.

# SUMMARY

Phthalylsulfathiazole was fed for short periods of time (8-17 days) to 5 human subjects on carefully controlled diets, and the effect upon the output of B-vitamins was determined, together with associated changes in bacterial counts of the feces. The fecal excretion of *L. casei* factor dropped to about 10% of previous values; the excretion of biotin also decreased, but not to as great a degree as did the *L. casei* factor. Upon removal of the drug, the *L. casei* factor and biotin content of the feces increased. No decisive decrease was found in any of the urinary vitamin excretion levels. Bacteriological findings included a marked and immediate decrease in numbers of coliform organisms, with a lesser effect upon the numbers of streptococci and lactobacilli.

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# Creatinase Activity of a Strain of *Pseudomonas*

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Received February 17, 1947

## INTRODUCTION

In a previous study one of us (HHB) described the production of creatine-creatinine destroying enzymes from human urine (1). Bacteriologic cultivation revealed the presence of organisms closely resembling *Pseudomonas aeruginosa* in such urine. The effect of this strain upon creatine and creatinine and some of their derivatives will be reported in this paper.

## EXPERIMENTAL

In order to obtain large numbers of bacteria they were grown on a medium containing 2% creatinine, 2% agar and 5% urine by volume. After incubation for 48 hours at 37°C., the bacteria were washed off with sterile physiological saline and centrifuged at high speed. This procedure was repeated twice with distilled water. The number of sedimented organisms was estimated by comparing suspensions of known volumes of bacteria in 10 ml. of distilled water with standardized barium sulfate suspensions using a photoelectric cell.

The washed bacteria were incubated with the solutions of the Jaffe-reactive substances, centrifuged, and the almost clear supernatant was used in all determinations. At the same time, control suspensions of the bacteria in distilled water were run. Creatine and creatinine were determined as usual with alkaline picrate before and after autoclaving the sample with HCl. Color intensities were measured with a Cenco-Sheard photometer. The same technique was used in the determination of glycoxyamidine and hydantoin since these substances also give the Jaffe reaction with alkaline picrate. Glycoxyamidine was estimated 30 minutes after adding the alkali, and hydantoin after 24 hours. Urea was determined with urease followed by addition of sodium carbonate and aeration of the ammonia into *N*/10 HCl, ammonia by addition of sodium carbonate and aeration into *N*/10 HCl, and carbon dioxide by liberation with acid in the Van Slyke apparatus.

## RESULTS

Several experiments were run with the organism and different substances under study. In order to save space a typical experiment of each type will be described here.

Fifty mg. each of creatinine, creatine, glycoxyamidine and hydantoin, in separate flasks containing 25 ml. of distilled water, were incubated at 37°C. for 24 hours with 0.6 ml. of a "creatinase" solution representing a suspension of  $1.5 \times 10^{10}$  *Pseudomonas* organisms. An equivalent suspension of the organisms and control solutions of the different substances in distilled water were incubated separately for the same length of time. The results obtained are listed in Table I.

TABLE I  
*Decomposition of Creatinine and Related Compounds  
by Pseudomonas "Creatinase"*

Substance	Amount added	Amount found in controls after incubation		Amount found after "creatinase" action		Amount decomposed per hour	
		Total Jaffe	Preformed Jaffe	Total Jaffe	Preformed Jaffe	Total Jaffe	Preformed Jaffe
	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Creatinine	50	50	50	0	0	2.1	2.1
Creatine	50	40	0	0	0	1.7	0.0
Glycoxyamidine	50	—*	50	—*	0	—*	2.1
Hydantoin	50	50	50	50	50	0.0	0.0

\* Not determined because of large scale destruction of glycoxyamidine on autoclaving.

Creatine, creatinine and glycoxyamidine were completely destroyed. Hydantoin was not attacked. About 10 mg. of creatine were destroyed in the control flasks without bacteria. The destruction of glycoxyamidine is not in conflict with the evidence obtained by one of us (HHB) (1) in a former study, in which it was shown that the "creatinine enzyme" (a group of organisms obtained from human urine) was unable to decompose glycoxyamidine. In this case the activity of the enzyme depended upon the growth of the organisms on the substrate, while in the present study no growth occurred. Glycoxyamidine was not broken down to any measurable extent because the organisms could not utilize it as the sole source of food, and the same was found to be true for *Pseudomonas* in the present study. Also, in another previous study (2) practically all of the glycoxyamine and glycoxyamidine in the liver extracts was destroyed by the experimental technique used before and during the action of the enzymes.

**TABLE II**  
*End Products of Decomposition of Creatinine and Creatine  
 by Pseudomonas "Creatinase"*

Substance	Amount added	Urea		Ammonia		Carbon dioxide	
		Theory	Found	Theory	Found	Theory	Found
Creatinine	mg. 50	mg. 26.5	mg. 26.3	mg. 7.5	mg. 5.9	mg. 19.5	mg. 11.0
Creatine	50	18.5	18.4	5.2	4.8	13.6	6.2

In Table II the theoretical values were calculated from the amounts of creatine and creatinine decomposed (Table I). It was assumed that 1 mole of urea, ammonia and carbon dioxide were formed from 1 mole of each of the two substances. This proved to be true in the case of urea, and fairly good agreement was obtained in the case of ammonia, while carbon dioxide was not produced in the required amounts.

It would seem from the above table that one molecule of urea is split off from a creatine or creatinine molecule. The residue, possibly sarcosine, is further broken down to ammonia and carbon dioxide. To test the likelihood of this assumption a solution of 50 mg. of sarcosine in distilled water was incubated with the *Pseudomonas* suspension for 48 hours with a quantitative production of ammonia. Urea itself is slowly decomposed by the suspension.

pH values from 4.5 to 9 and incubation at 28°C. instead of 37°C. did not interfere with the enzyme activity. In the presence of toluene the

**TABLE III**  
*Partial Inhibition of the Decomposition of Creatinine by Pseudomonas  
 "Creatinase" in the Presence of 2% Toluene*

	Amount of creatinine added	Amount found after "creatinase" action	
		Total	Preformed
Toluene	mg. 50	mg. 37	mg. 26
No toluene	50	0	0

enzyme is partly inhibited but the values for the "total amount found" are somewhat larger than those for the "preformed amount found" (Table III). Similar results were obtained throughout the course of the

studies whenever intermittent readings of creatinine breakdown by the enzyme were made. Since the difference between the total and pre-formed amounts can only be creatine one is led to believe that creatinine was hydrated to creatine.

Attempts to extract the enzyme from the bacterial cells by means of distilled water were unsuccessful. Organisms suspended in the water for 48 hours displayed normal enzyme activity when centrifuged and incubated with creatinine, whereas the supernatant after filtration through a Seitz filter proved to be completely inactive toward creatinine.

### DISCUSSION

The experimental data reported above indicate that the enzyme obtained from *Pseudomonas* organisms first converts creatinine into creatine, and then proceeds to split the creatine molecule into urea and, presumably, sarcosine. Glycocyamidine is also readily destroyed by the enzyme. Its conversion into glycocyamine and subsequent slow breakdown analogous to that of creatine are suggested by the results of some unpublished experiments. Hydantoin is not attacked at all. Maximum enzyme activity seems to depend upon the presence of a  $-C-N-$  grouping such as that found in creatine and creatinine.



Among the decomposition products, ammonia and carbon dioxide are most probably derived from the sarcosine part of the molecule of creatine and creatinine. This seems to be in agreement with the results of Webster and Bernheim (3) on the oxidation of amino acids by *Pseudomonas aeruginosa*. While sarcosine was not studied, experiments performed with closely related amino acids, such as alanine and glycine, resulted in the production of ammonia and carbon dioxide in addition to unidentified oxidation products.

The organisms may produce two active enzymes, a creatinine hydase and a creatine hydrolase, but sufficient data have not yet been obtained to justify this conclusion. On the other hand, a single enzyme "creatinase" may be capable of transforming creatinine into creatine and then splitting the latter into urea and sarcosine.

## SUMMARY

1. A "creatinase" solution was obtained from *Pseudomonas* organisms isolated from human urine.
2. Its activity toward creatine, creatinine, glycocyamidine, and hydantoin was determined.
3. End products of the decomposition of creatine and creatinine were found to include urea, ammonia, and carbon dioxide.
4. The enzyme cannot be extracted from the bacterial cell by means of distilled water.
5. The enzyme is partially inhibited in the presence of toluene.
6. The nature of the enzyme and its mode of action are discussed.

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# Inhibition of the Action of Dopa Decarboxylase

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Received April 15, 1947

## INTRODUCTION

In a previous communication (1) it was reported that desoxyypyridoxine phosphate exerted an inhibitory action on tyrosine decarboxylase derived from *Strep. faecalis* R. Since the end in view in the investigation was the application of the findings to the problem of renal hypertension, it was considered desirable to amplify the study to include an animal decarboxylase. For this purpose dopa decarboxylase was chosen both because of the pressor effect produced by injections of dopa in hypertensive individuals (2) and because of the fact that the relatively large amounts of CO<sub>2</sub> produced by the action of the enzyme make it possible to study its action manometrically.

## EXPERIMENTAL

Dopa decarboxylase was prepared according to the method of Holtz and Credner (3). Rat kidney was ground with sand in 5 volumes of *M*/20 phosphate buffer, pH 6.5. The suspension was centrifuged and the supernatant assayed in the Warburg apparatus. Each cup contained 2 cc. of the enzyme preparation and 0.5 cc. of a solution of the displacing agent in water. The side bulb contained 3 mg. of *l*(-)-dopa dissolved in 0.5 cc. of water. The manometers were filled with nitrogen and equilibrated at 37°C. for 15 minutes. At the end of this time the substrate was tipped into the center compartment and the reaction was run for 15 minutes. It was found that no CO<sub>2</sub> was evolved after this period. At the end of this time the average volume (in mm.<sup>3</sup>) of CO<sub>2</sub> evolved in the control cups was compared with the average evolved in the cups containing the displacing agents. The inhibition was calculated from the equation:

$$\frac{[\text{CO}_2 \text{ (control)} - \text{CO}_2 \text{ (experimental)}] \times 100}{\text{CO}_2 \text{ (control)}} = \% \text{ inhibition.}$$

The values for inhibition presented below represent average values for at least 3 runs in each case. Each experiment gave 2 values for the controls and 4 values for the experimental cups, so that each figure for inhibition represents an average of at least 6 separate controls and 12 experimental results.

Desoxyypyridoxine phosphate caused no inhibition of the action of dopa decarboxylase. It was found, however, that two folic acid displacing agents, 7-methylfolic acid [N-(4-((2-amino-4-hydroxy-7-methyl-6-pteridyl)-methyl)-amino)benzoyl)-l-(+)-glutamic acid] (4), and the aspartic acid analogue of folic acid [N-(4-((2-amino-4-hydroxy-6-pteridyl)-methyl)-amino)-benzoyl)-aspartic acid] (5), were effective as inhibitors of the decarboxylation of l(-)-dopa.

7-Methylfolic acid at a concentration of 300  $\gamma$ /cc. caused an inhibition of 50%; at 30  $\gamma$ /cc. it caused an inhibition of 25%. A representative set of experimental figures is presented below.

TABLE I

*Inhibition of the Action of Dopa Decarboxylase by 7-Methylfolic Acid*  
Evolution of CO<sub>2</sub> (mm.<sup>3</sup>)

Control	Control	1	2	3	4
14.6	15.0	11.2	11.4	10.8	10.7

Average (control)—14.8  $\pm$  0.2

Average (experimental)—11.0  $\pm$  0.2

$$\% \text{ Inhibition} = \frac{14.8 - 11.0}{14.8} \times 100 = 25.7$$

Time of experiment—15 minutes

Cups 1,2,3 and 4 contained 30  $\gamma$ /cc. of 7-methylfolic acid

The aspartic acid analogue of folic acid was slightly less effective. At a concentration of 300  $\gamma$ /cc. it caused an inhibition of 50%; at 30  $\gamma$ /cc., 15%.

That this inhibitory effect was due to a displacement of folic acid was shown by the fact that addition of folic acid to the reaction mixture nullified the effect of methyl folic acid. When folic acid was added at 10–100 times the concentration of the methylfolic acid, no inhibition was obtained.

The action of tyrosine decarboxylase was inhibited only by much higher concentrations of methylfolic acid than were necessary to inhibit the action of dopa decarboxylase. There was no inhibition at a concentration of 300  $\gamma$ /cc. A concentration of 3000  $\gamma$ /cc., however, caused an inhibition of 40%. The method used for the investigation of tyrosine decarboxylase was that reported previously (1).

A comparison of the action of methylfolic acid on these two enzymes is given below in tabular form.

TABLE II  
*Inhibitory Effect of 7-Methylfolic Acid on Tyrosine  
Decarboxylase and Dopa Decarboxylase*

Concentration ( $\gamma$ /cc.)	Per cent Inhibition	
	Dopa Decarboxylase	Tyrosine Decarboxylase
30	25	—
300	50	0
3000	60	40

The fact that desoxypyridoxine phosphate, which has been shown to be an effective displacer of pyridoxal phosphate in the tyrosine decarboxylase system, has no effect on dopa decarboxylase, suggests that pyridoxal phosphate is not the coenzyme of the latter. However, the effectiveness of two folic acid displacers as inhibitors of dopa decarboxylase would seem to point to folic acid, or some derivative of folic acid, as having a coenzyme function. These findings provide, in any event, a point of departure for the study of the decarboxylation of dopa in the animal body.

The inhibitory action of methylfolic acid on tyrosine decarboxylase, occurring, as it does, only at extremely high concentrations of methylfolic acid, cannot be considered to be a true displacement.

### SUMMARY

Desoxypyridoxine phosphate does not inhibit the action of dopa decarboxylase.

7-Methylfolic acid and the aspartic acid analogue of folic acid are effective as inhibitors of dopa decarboxylase.

The inhibitory action of methylfolic acid on dopa decarboxylase is nullified by folic acid.

Methylfolic acid inhibits tyrosine decarboxylase only in very high concentrations.

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# Phenolsulfatase Activity

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Received May 5, 1947

## INTRODUCTION

A study of some of the reaction kinetics of phenolsulfatase activity is presented. As a convenient and reliable source of enzyme, a preparation from *Aspergillus oryzae*<sup>1</sup> was used. Enzyme activity was measured by the amount of phenol liberated from potassium phenylsulfate in a specified time at the optimum pH and temperature using a modification of Folin's colorimetric micromethod for the determination of phenol.

## EXPERIMENTAL

### *Effect of pH*

The optimum pH for phenolsulfatase activity has been stated to be variously 5, 6, 7 and 9 (1, 2, 3). The phenolsulfatase activity of 0.5 ml. of a 5% takadiastase solution was measured at 37.5°C. using as buffer-substrate 10 ml. portions of solutions of 0.005 *M* potassium phenylsulfate in 0.1 *M* sodium acetate-acetic acid of varied pH. At pH 8.6, the substrate was buffered in 0.1 *M* ammonium chloride-ammonium hydroxide. The pH of the buffer-substrate solution was measured electrometrically at room temperature (25°C.). Addition of the enzyme solution caused little, if any, alteration. The incubation period in each case was 3 hours.

The pH-activity curve (Fig. 1) showed a single, sharp maximum between pH 6.1 and 6.4. The optimum substrate concentration at pH 6.2 subsequently was found to be 0.02 *M*, and the optimum pH was again determined using as buffer-substrate 0.02 *M* potassium phenylsulfate in 0.2 *M* sodium acetate-acetic acid. A similar curve was obtained, and a buffer-substrate solution having pH 6.2 (at 25°C.) gave maximum activity.

<sup>1</sup> Takadiastase (Parke, Davis and Company).

*Effect of Substrate Concentration*

The phenolsulfatase activity of 0.5 ml. of a 5% takadiastase solution was measured at 37.5°C. using 10 cc. of buffer-substrate solutions of pH 6.2 with concentrations of potassium phenylsulfate varying from 0.0004 *M* to 0.02 *M* in 0.2 *M* sodium acetate-acetic acid. The incubation period in each case was 3 hours.

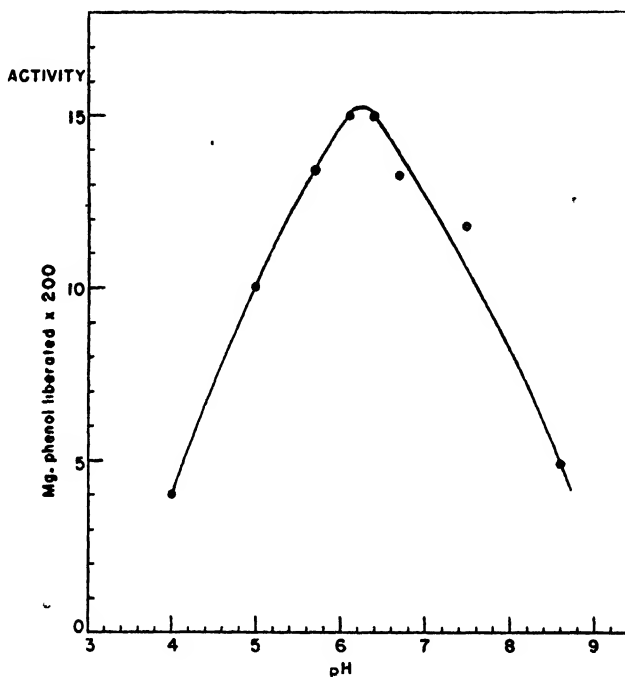


FIG. 1. Variation of phenolsulfatase activity with pH.  
Incubation for 3 hours at 37.5°C.

The increase in rate of reaction with increasing substrate concentration reached a maximum with a buffer-substrate solution containing 0.02 *M* potassium phenylsulfate<sup>2</sup> (Fig. 2, Curve I). At this concentration, 0.47% of the substrate was hydrolyzed in the 3-hour period under the conditions described. Higher concentrations interfered with phenol determinations by causing precipitation during color development.

<sup>2</sup> Actual substrate concentration after addition of the enzyme solution was 5% less in each case.

Two-hundredth *M* potassium phenylsulfate in 0.2 *M* sodium acetate-acetic acid at pH 6.2 was used as buffer-substrate solution for maximum activity in subsequent work. Activity plotted against the logarithm of substrate concentration indicated that half-maximum activity was attained at a substrate concentration approximately  $9 \times 10^{-4}$  *M*. The ratio, substrate concentration/activity,  $(S)/V$ , plotted against substrate concentration (*S*) gave a straight line (Fig. 2, Curve II), and the Michaelis constant, calculated by the least squares method from the linear equation  $(S)/V = (S)/V_{\max} + K_s/V_{\max}$  (4), was found to be  $9.7 \times 10^{-4}$  *M* under the conditions of this experiment.

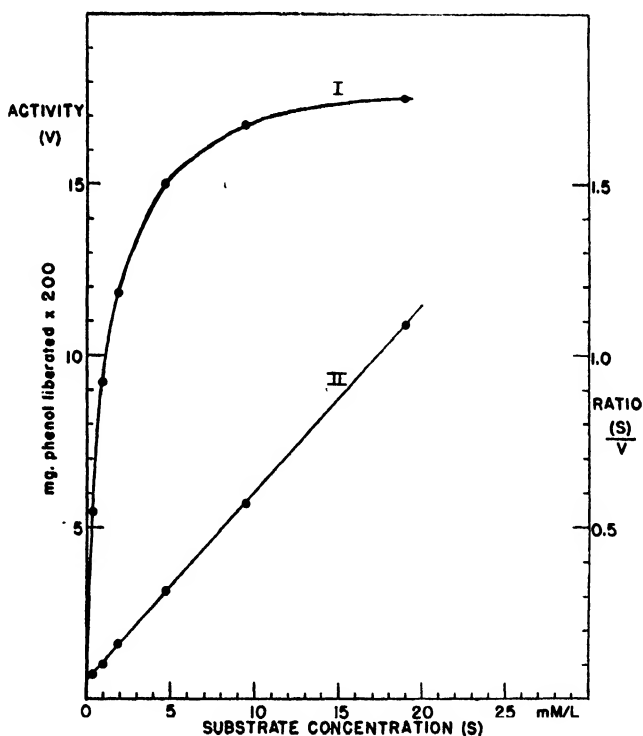


FIG. 2. Effect of substrate concentration on phenolsulfatase activity.  
Incubation for 3 hours at 37.5°C. and pH 6.2

I. Activity (*V*) plotted against substrate concentration (*S*); II. Ratio,  $(S)/V$  plotted against (*S*).



### *Effect of Temperature*

The phenolsulfatase activity of 0.5 ml. of 5% takadiastase was measured at temperatures from 0° to 80°C. Incubation was for 3 hours at pH 6.2, with 0.02 *M* potassium phenylsulfate in 0.2 *M* sodium acetate-acetic acid as buffer-substrate.

Maximum activity was noted at 50°C. (Fig. 3). Inhibition of activity at 0°C. was reversible; 5% takadiastase kept at 0°C. for one hour retained 100% of its activity when measured at 50°C. After one hour at 80°C., however, the enzyme solution was inactive. There was also total loss of activity after five minutes at 100°C. Temperature coefficients noted for 10° intervals starting at 10°C. were 1.9, 1.7, 1.9 and 1.2.

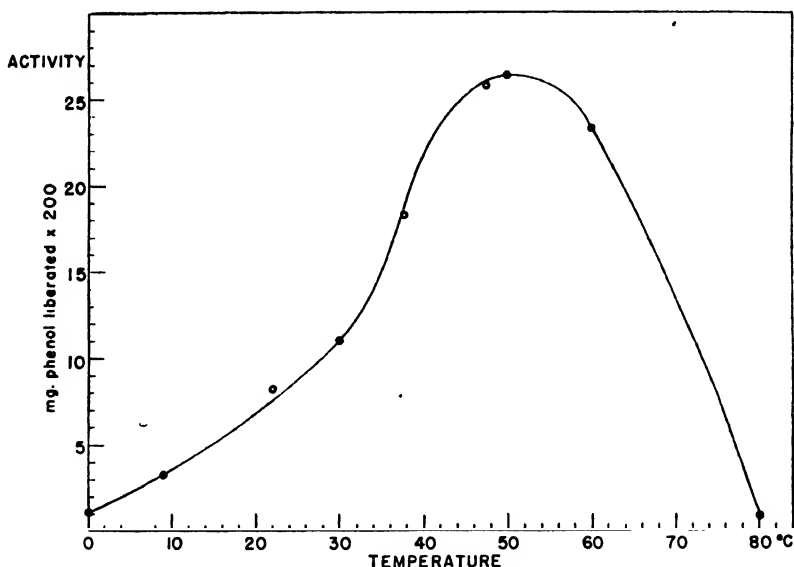


FIG. 3. Effect of temperature on phenolsulfatase activity. Incubation for 3 hours at pH 6.2 and optimum substrate concentration.

### *Effect of Time of Incubation*

Phenolsulfatase activity was measured at pH 6.2 with 0.02 *M* potassium phenylsulfate in 0.2 *M* sodium acetate-acetic acid as buffer-substrate and incubation periods varying from one to four hours.

The activity of a 5% takadiastase solution was measured at 50°C. (Fig. 4, Curve I) and at 37.5°C. (Fig. 4, Curve II), and the activity of

2% takadiastase also was measured at 50°C. (Fig. 4, Curve III). In each case there was a noticeable decrease in the rate of activity after the first hour. After this time the rate of activity tended to remain more constant. This effect was less apparent at 37.5°C. than at 50°C. At 50°C., however, with 2% and 5% takadiastase, proportionality between enzyme concentration and activity was evident at the same time

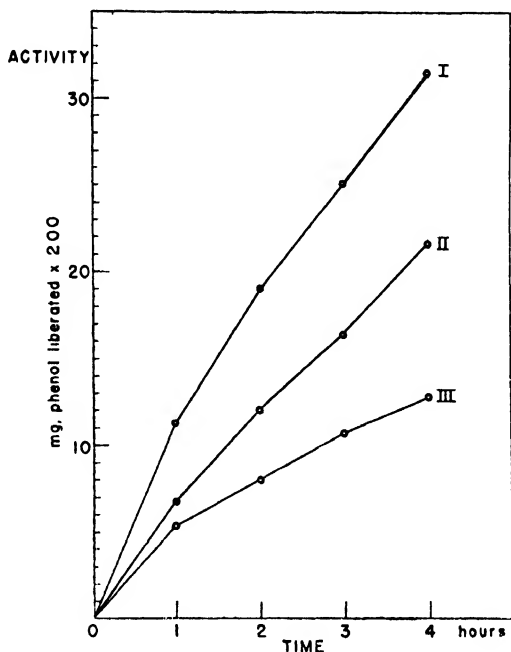


FIG. 4. Variation of phenolsulfatase activity with time of incubation at pH 6.2 and optimum substrate concentration.

I. 5% takadiastase at 50°C.; II. 5% takadiastase at 37.5°C.; III. 2% takadiastase at 50°C.

intervals (Fig. 4, Curves I and III). These activities have been replotted as functions of enzyme concentration and are included with the curve showing relationship of activity to enzyme concentration (Fig. 5).

*Effect of Enzyme Concentration*

Under conditions previously found to give maximum activity, the phenolsulfatase activity of 0.5 ml. of varied dilutions of a 10% takadiastase solution was measured at pH 6.2 using 0.02 *M* potassium phenylsulfate in 0.2 *M* sodium acetate-acetic acid as buffer-substrate with incubation at 50°C. for one hour.

The data show a direct relationship between enzyme concentration and rate of reaction (Fig. 5, Curve I). Data from Fig. 4 also were in-

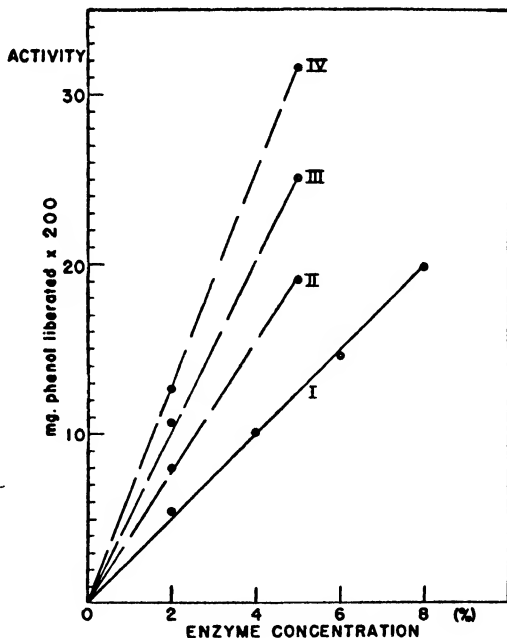


FIG. 5. Variation of phenolsulfatase activity with enzyme concentration at pH 6.2 and optimum substrate concentration at 50°C. Enzyme concentration is expressed as the per cent takadiastase in the solution tested.

I. One-hour incubation; II. Two-hour incubation (from Fig. 4); III. Three-hour incubation (from Fig. 4); IV. Four-hour incubation (from Fig. 4).

cluded in Fig. 5 for comparison and indicate a similar relationship. Thus, Curves II, III, and IV (Fig. 5) represent phenolsulfatase activity of 2% and 5% takadiastase after 2, 3 and 4 hours incubation at 50°C., respectively.

### Determination

Folin's reagent for the determination of phenol was used essentially as in the Gutman and Gutman modification (5) of the King and Armstrong procedure (6) for determining phenol in the phosphatase method. Optimum conditions for enzyme activity were selected according to the results of the preceding experiments, and a few necessary changes in procedure were made. All determinations were made in duplicate.

**Reagents.** 1. Buffer-substrate (0.02 *M* potassium phenylsulfate (7, 8) in 0.2 *M* sodium acetate-acetic acid at pH 6.2).<sup>3</sup> Equal parts of solutions A and B were mixed as needed and the pH checked.

Solution A. 2.12 g. of potassium phenylsulfate were dissolved in water and diluted to 250 ml. (Kept cold.)

Solution B. 32.8 g. sodium acetate (C.P. anhydrous) were dissolved in water and diluted to one liter. 30 ml. of 0.4 *M* acetic acid were added and the pH checked. If necessary, the pH was adjusted to 6.2 with NaOH or acetic acid.

2. Phenol reagent of Folin and Ciocalteu (9, 10). This was diluted for use as needed, one volume with two of water.

3. Phenol standard. A dilute phenol standard containing exactly 10 mg. of phenol/100 ml. was made from the standardized stock solution (10). (Kept cold.)

4. Standard phenol solution plus reagent. One ml. of the dilute phenol standard plus 3 ml. of diluted phenol reagent was made to a volume of 10 ml. in a graduated Klett tube. This solution was prepared for use just before color development.

**Procedure.** Four test tubes, 2 for control (C) and 2 for test (T) each containing 10 ml. of buffer-substrate solution, and 2 test tubes (B) each containing buffer only (solution B and water, 1:1) were kept in a water bath controlled at 50°C. for 5 minutes. After this time 4 tubes (T and B) were removed, exactly 0.5 ml. of solution (or serum) to be tested was added to each, and all were replaced in the water bath at 50°C. after careful mixing of the contents and stoppering. One hour from the time the enzyme solution was added to set (T), or after the time interval used if longer incubations were found necessary, the 4 tubes containing the enzyme (T and B) were removed from the water bath and 4.5 ml. of diluted phenol reagent were added at once to (T) and to (B) and the contents of each tube mixed. One-half ml. of enzyme solution, immediately followed by 4.5 ml. of diluted phenol reagent, was added to each control tube (C) and mixed. All tubes were then centrifuged or filtered.<sup>4</sup> Exactly 6 ml. of each filtrate were transferred to test tubes for color development. These tubes, with the standard phenol solution plus reagent (No. 4) prepared shortly before, were placed in a 37.5°C. water bath for five minutes,<sup>5</sup> removed, and 20% sodium carbonate

<sup>3</sup> Use of barium acetate buffers was discarded when, during preliminary experiments, barium was found to interfere by causing precipitation with the phenol reagent.

<sup>4</sup> With many of the enzyme preparations used the amount of protein present was too small to give precipitation or turbidity.

<sup>5</sup> With substrate concentration 0.02 *M* or higher, precipitation will occur on alkalization of the control (C) filtrates if carried out at room temperature. This was not noted with lower substrate concentrations and was influenced greatly by room temperature. At 37.5°C. precipitation did not occur in these filtrates on alkalization.

added to each tube, 2.5 ml. to the standard tube, 1.5 ml. to each of the others. The contents of each were mixed immediately and all tubes were replaced in the water bath (37.5°C.) for 5 minutes to develop color. The tubes were then removed and, after standing 30 minutes at room temperature, were read in a Klett-Summerson photoelectric colorimeter (Filter 42).

*Calculation.* The difference ( $D$ ) between the reading of the test and control filtrates represents the phenol liberated from the substrate by the enzyme in the time interval used.\* The reading of the standard ( $S$ ) represents 0.1 mg. phenol. Mg. phenol liberated by the sample taken is given by the following equation.

$$\text{mg. phenol} = \frac{D}{S} \times 0.1 \times \frac{7.5}{12.5} \times \frac{15}{6} = \frac{D}{S} \times 0.15.$$

If the sample were 0.5 ml. and calculation to 100 ml. of enzyme solution (or serum) were desired, the above equation becomes  $\frac{D}{S} \times 30$  and represents mg. phenol liberated/100 ml. of solution tested during the time interval used. In the following paragraph phenolsulfatase activity is expressed as units. For this purpose a unit is defined as that degree of phenolsulfatase activity which, at 50°C., will liberate 1 mg. of phenol in 1 hour from 0.02  $M$  potassium phenylsulfate in 0.2  $M$  sodium acetate-acetic acid at pH 6.2.

### *Phenolsulfatase Activity*

A number of enzyme preparations similar to takadiastase were studied (Table I). It will be seen that all active preparations studied had greater activity than the takadiastase preparation used in the experiments presented, and that the greatest activity was obtained with Mylase P, a concentrated, highly active preparation of many enzymes of fungal origin. Enzyme preparations, available in the laboratory, tested and found to have no phenolsulfatase activity, in-

\* It was noted that the blank values (no enzyme activity) obtained with all controls agreed very well in most cases and that the color value of the control (C) was due to chromogenic material in the enzyme solution and not to hydrolysis of the substrate at the pH, temperature and time used, provided the buffer-substrate solution originally gave no color with phenol reagent. It is necessary, however, to take the precaution of using the additional control (B) when investigating new material, to ensure that chromogenic material does not arise from the enzyme solution itself during incubation. Such a condition was noted as occurring with certain glycerol extracts of liver and kidney, and in these cases control values obtained in this manner were higher than buffer-substrate controls with enzyme added after incubation. This was not the case with enzyme preparations reported in this paper, or with serum, under the conditions used. When it has been established that control (B) is not higher than control (C) for the material being studied, it is possible to dispense with control (B). In all work reported in this paper, both controls (C) and (B) were used in each determination. If exact agreement did not occur, the higher value of the two sets (usually difference was slight, if any) was used in calculation of enzyme activity.

TABLE I

*Phenolsulfatase Activity of Enzyme Preparations Similar to Takadiastase*

0.5 ml. of 2% enzyme solution with one hour incubation at 50°C. was used in each case.

Enzyme preparation	Phenolsulfatase activity units/g.
Takadiastase <sup>a</sup> (Parke, Davis and Co.)	2.6
Takadiastase <sup>b</sup> (Parke, Davis and Co.)	3.7
Polidase S (Schwarz Laboratories)	6.3
Clarase (Takamine Laboratories)	8.3
Diastase, Vera (Eimer and Amend)	9.2
Mylase P (Wallerstein Laboratories)	19.4
Diastase of malt (Merck)	0.0
Amylopsin (Eimer and Amend)	0.0

<sup>a</sup> Preparation used throughout previous experiments reported in this paper.<sup>b</sup> Preparation from different, unopened stock.

cluded diastase of malt (Merck), amylopsin (Eimer and Amend), pepsin (Merck), trypsin (Eimer and Amend), pancreatin (Merck), rennet (Armour), yeast extract (Difco) and jack bean meal (Arlco). The method may thus be used in the selection of active preparations, and to follow activity in the course of concentration and purification of the enzyme. The activity curves presented illustrate that the method may be found useful in the study of the kinetics of phenolsulfatase activity with purer preparations.

Since phenolsulfatase activity has been demonstrated to be present in certain human organs (11) we have examined human sera for phenolsulfatase activity with this method. No activity was obtained with normal human sera using either 0.5 ml. or 1 ml. at either 37.5°C. or 50°C. for incubation periods up to 18 hours.

### SUMMARY

Curves showing the effect of pH, substrate concentration, temperature, incubation time, and enzyme concentration on the activity of the phenolsulfatase of takadiastase are presented. Optimum activity was obtained at 50°C. with 0.02 *M* potassium phenylsulfate in 0.2 *M* sodium acetate-acetic acid buffer (pH 6.2 at 25°C.).

A method suitable for the determination of phenolsulfatase activity in small amounts of material by measurement of the amount of phenol liberated from potassium phenylsulfate with comparatively short incubation periods is presented.

Phenolsulfatase activity of a number of enzyme preparations was determined.

No activity was noted in human serum with this method at either 37.5°C. or 50°C., for periods up to 18 hours.

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## A New Antibiotic Produced by a Strain of *Streptomyces lavendulae*

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Received May 13, 1947

### INTRODUCTION

The actinomycetes are the source of a number of different antibiotics among which have been described actinomycin A and B (1), proactinomycin (2), mycetin (3), streptothricin (4), streptomycin (5), actinomycetin (6), micromonosporin (7) and streptin (8). To this list can be added another antibiotic, which was isolated and studied in our laboratories during the course of a survey of actinomycetes for antibiotic activity (9). This antibiotic, which we have chosen to designate tentatively as Antibiotic 136,<sup>1</sup> is produced by a strain of *Streptomyces lavendulae*, No. 136B, and is similar in its chemical properties to streptothricin. It differs from streptothricin, however, in its greater toxicity to mice and in its antibacterial spectrum.

### EXPERIMENTAL

#### *Production and Assay*

Beers with a high antibiotic titer were produced in 500 ml. shaker flasks and in 30 liter and 75 gallon fermenters. The culture medium contained 1.2% Hy-Case and 1.0% glucose in tap water and the pH was adjusted to 6.5-7.0. The medium was inoculated with spores and incubated with agitation at 23°C. for 3-4 days.

Assays of antibiotic activity were accomplished by either a broth dilution or an agar plate method. For the broth dilution assay, the antibiotic was diluted in tubes of broth (0.75% Bacto peptone, 0.25% yeast extract; pH 7.25) which were inoculated from a 24-hour old culture of *Staphylococcus albus* and incubated for 24 hours at 30°C.

<sup>1</sup> Workers at the University of Wisconsin have independently discovered an antibiotic which has many properties in common with our Antibiotic 136. Final decision on the identity or non-identity of the two compounds must await further comparisons on compounds of known purity.



The antibiotic titer was read as the highest dilution giving complete inhibition of the growth of the test organism. For the plate assay, agar cup plates were prepared, each with 20 ml. of the following medium: Difco yeast extract 3.0 g., Bacto peptone 6.0 g., Bacto beef extract 1.5 g.,  $K_2HPO_4$  2.0 g., NaCl 2.0 g., agar 20.0 g. and distilled water to 1,000 ml.; pH 8.0. Each plate was surfaced with 4 ml. of agar seeded with *Bacillus subtilis*. The diameters of the zones of inhibition were measured and recorded.

### *Extraction of Antibiotic Activity From Culture Filtrates*

A typical extraction was performed as follows. The beer which was harvested from three 30 liter fermenters was clarified by filtration with 2% Celite 545. The volume of the filtrate was 57 liters and its potency equaled 40,000 *Staph. albus* dilution units/ml. After adjustment of the pH to 2.5 with concentrated HCl, the beer was stirred for one-half hour with 300 g. of Darco G-60, filtered with 600 g. of Celite 545, and the filtrate adjusted to pH 7.5 with NaOH. The carbon removed most of the color but none of the antibiotic activity. The filtrate was stirred with 1,200 g. of Folin Decalso (Permutit according to Folin) for one-half hour and filtered. The permutit adsorbed 94% of the activity, which was then eluted by stirring twice with 300 ml. portions of a 10% aqueous  $NH_4Cl$  solution. The first eluate contained 58% and the second eluate 18% of the original activity. The  $NH_4Cl$  eluates were combined, adjusted to pH 7.4 with NaOH and stirred one-half hour with 240 g. of Darco G-60, and filtered. Only part of the activity was removed by one carbon treatment so the filtrate was stirred with another 240 g. of Darco G-60, and filtered. All of the remaining activity was removed by the second carbon treatment. Each of the two carbons was separately eluted twice at room temperature with a solution of 0.05 *N* HCl in 50% aqueous methanol (5.0 ml. of eluate/g. of carbon). The eluates from the first carbon contained 33% and the eluates from the second carbon contained 29% of the original activity. The carbon eluates were concentrated *in vacuo* until the solution contained 10% solids and then were filtered through sintered glass. To the filtrate were added 8 volumes of acetone. The precipitates which formed were dissolved in methanol (to make a 5-10% solution) and to this solution 8 volumes of acetone were added with vigorous shaking. A white precipitate was formed which was centrifuged, washed several times with acetone and dried under vacuum. The loss of activity in the acetone solution was of the order of 3% of the original. The eluates from the first carbon adsorption produced 10.0 g. of product (Antibiotic 136) and the eluates from the second neutral carbon adsorption yielded 3.0 g. of dried product (Fraction B).

### *The Production of Two Antibiotics by Culture 136B*

When the antibiotic activities of the first carbon and second carbon fractions were determined and compared, it was apparent that the two fractions were different from each other. The first carbon fraction contained an antibiotic substance which differed in its antibacterial spectrum from all previously described antibiotics and it is to this fraction that we have given the name Antibiotic 136. The preparation of Anti-

biotic 136 which has been used for all studies reported in this paper is not crystalline and it contains an unknown amount of impurities.

The second carbon fraction, which will be referred to as Fraction B, contained an antibiotic substance which has been crystallized and which appears to be streptothricin. Fraction B will be briefly discussed at the end of this paper.

### *Adsorption and Elution Studies of Antibiotic 136*

A variety of solutions were studied to determine their efficacy in eluting Antibiotic 136 from Decalso. Those first tried were: 0.05 *N* HCl in H<sub>2</sub>O, methanol, 50% aqueous methanol, 0.05 *N* HCl in methanol, 0.05 *N* HCl in 50% aqueous methanol, 5% aqueous pyridine, 5% pyridine hydrochloride in H<sub>2</sub>O (pH 1.5–2), and 5% NH<sub>4</sub>Cl in 0.05 *N* HCl. Only the last two solutions were effective as eluting agents. Since it appeared that an ion exchange phenomenon was involved in the elution of the antibiotic from Decalso, various salt solutions were tried as eluting agents (Table I). A further study was then made of the

TABLE I  
*The Effectiveness of Various Salt Solutions in  
Releasing Antibiotic 136 from Decalso*

Salt solution	Percentage of activity released by salt solution
10% NH <sub>4</sub> Cl	90.0
10% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	52.5
10% NH <sub>4</sub> NO <sub>3</sub>	70.0
10% NaCl	37.5
10% CaCl <sub>2</sub>	25.0
10% AlCl <sub>3</sub>	35.0
10% MgSO <sub>4</sub>	7.0
10% ZnSO <sub>4</sub>	3.0
2% Pyridine-HCl, pH 2.5	20.0
3% Aniline-HCl, pH 4.0	10.0

effect of concentration of NH<sub>4</sub>NO<sub>3</sub> upon its ability to elute the antibiotic (Table II). It was found that Decalso, after elution with salt solutions and washing with water, can be reused with no apparent loss in its effectiveness in adsorbing Antibiotic 136.

In addition to Decalso, Antibiotic 136 could be adsorbed on superfiltrol at pH 7.0, Amberlite IR-100 (The Resinous Products and Chemical Company), Zeo Karb "H" (The Permutit Company), Ionac C-284 (American Cyanamide Company), and acti-

TABLE II

*The Effect of Different Salt Concentrations in  
Releasing Antibiotic 136 from Decalco*

Salt solution	Percentage of activity released from decalco
5.0% $\text{NH}_4\text{NO}_3$ -0.05 N $\text{HNO}_3$	56
2.5% $\text{NH}_4\text{NO}_3$ -0.05 N $\text{HNO}_3$	18
1.0% $\text{NH}_4\text{NO}_3$ -0.05 N $\text{HNO}_3$	less than 2
0.5% $\text{NH}_4\text{NO}_3$ -0.05 N $\text{HNO}_3$	less than 1

vated alumina (Merck's according to Brockman or Activated Alumina F-1 of the Aluminum Ore Company). Antibiotic 136 was eluted from superfiltrol in nearly quantitative yields by acid solutions (pH 1.5-2.0) of the sulfates or hydrochlorides of pyridine, diethylamine, ethylenediamine, morpholine, or brucine.

#### *Miscellaneous Chemical Properties of Antibiotic 136*

The titration curve for Antibiotic 136 indicates that there are present both strong and weak basic groups. Antibiotic 136 is readily precipitated by basic precipitants and shows no acidic properties. The hydrochloride salt as prepared above is soluble in methanol and readily precipitated with acetone, but on further purification of the antibiotic preparation the precipitation by acetone is less complete. The sulfate of Antibiotic 136 can be precipitated from aqueous solutions with methanol. Antibiotic 136 can also be precipitated with silver at pH 7.4-9.5. Only a small part of the total antibiotic substance is precipitated by basic lead acetate or mercuric acetate at pH 6.5. Flavianic acid and picrolonic acid precipitate the antibiotic from solution, but these precipitants did not yield a crystalline product. To the present time, we have been unable to isolate crystalline derivatives of Antibiotic 136.

#### *Stability of Antibiotic 136*

The degree of stability of a 0.5% solution of Antibiotic 136 at 100°C. at different pH values is recorded in Table III. The highest degree of thermostability is exhibited at a pH of 2.0.

#### *Antibacterial Activity of Antibiotic 136*

In determining the bacterial spectrum, Antibiotic 136 was diluted in tubes of one of three different broth media: (1) 0.75% Bacto peptone, 0.25% Difco yeast extract, distilled water, pH 7.25, (2) 1.5% Difco proteose peptone No. 3, 0.1% Difco yeast

TABLE III

*The Stability of Antibiotic 136 in Aqueous Solutions*

pH of solutions	Percentage of activity left after heating at 100°C.	
	1 hour	2 hours
8.0	10	4
6.0	61	50
4.0	75	65
2.0	90	65
2.0 plus HCl added to make 0.05 N acid	50	30
2.0 plus HCl added to make 0.1 N acid	23	10

extract, 0.1% glucose, 0.1% NaCl, 0.1% K<sub>2</sub>HPO<sub>4</sub>, distilled water, pH 7.5, or (3) Long's synthetic medium at pH 7.6. *Mycobacterium tuberculosis* was inoculated into Medium No. 3 and *Brucella abortus*, *Eberthella typhosa*, and *Streptococcus hemolyticus* were grown in Medium No. 2. Medium No. 1 was used for all remaining bacteria. The bacterial spectra of the 137-NB-3 preparation of Antibiotic 136 are presented in Table IV.

TABLE IV

*Bacterial Spectrum of Antibiotic 136*

Organism <sup>1</sup>	Activity <sup>2</sup>	Organism <sup>1</sup>	Activity <sup>2</sup>
<i>M. tuberculosis</i>	300,000	<i>S. schottmulleri</i> ATCC 9149	14,000,000
<i>S. marcescens</i>	800,000	<i>A. aerogenes</i> ATCC 8308	16,000,000
<i>S. hemolyticus</i>	1,000,000	<i>A. aerogenes</i>	18,000,000
<i>B. cereus</i> ATCC 9139	1,000,000	<i>B. subtilis</i> ATCC 8243	20,000,000
<i>Ps. fluorescens</i>	1,200,000	<i>B. subtilis</i> ATCC 6051	20,000,000
<i>Ps. aeruginosa</i> ATCC 9027	1,200,000	<i>B. polymyxa</i> ATCC 8523	20,000,000
<i>P. vulgaris</i> ATCC 220	3,000,000	<i>B. mesentericus</i>	30,000,000
<i>H. anomala</i> ATCC 8202	3,000,000	<i>S. aureus</i> ATCC 9144	30,000,000
<i>B. abortus</i>	3,000,000	<i>A. viscosus</i> ATCC 9036	40,000,000
<i>E. coli</i>	4,000,000	<i>S. suispestifer</i>	50,000,000
<i>B. subtilis</i>	4,000,000	<i>S. aureus</i> F.D.A. 209	50,000,000
<i>P. vulgaris</i>	5,000,000	<i>S. albus</i> ATCC 151	60,000,000
<i>S. enteritidis</i> ATCC 9118	6,000,000	<i>Phy. campestris</i> ATCC 7381	60,000,000
<i>E. typhosa</i>	7,000,000	<i>G. tetragen</i> ATCC 159	120,000,000
<i>B. mycoides</i>	10,000,000	<i>B. subtilis</i>	140,000,000
<i>S. gallinarum</i>	12,000,000	<i>S. citreus</i> ATCC 395	180,000,000
<i>E. coli</i> ATCC 26	14,000,000		

<sup>1</sup> Those cultures which were not obtained from the American Type Culture Collection are part of The Upjohn Company's collection.

<sup>2</sup> The activity is the highest dilution at which 1 g. of preparation 137-NB-3 will completely inhibit the growth of the test organism.

The antifungal activity of Antibiotic 136 against a number of fungal pathogens of man was determined by diluting the antibiotic in agar and streaking the agar plates with spore suspensions of the fungi to be tested. The agar had the following composition: 0.5% Bacto peptone, 0.1% yeast extract, 1% dextrose, 2.0% agar, distilled water; and was adjusted to pH 8.0 with NaOH before autoclaving. The antifungal activity of preparation 137-NB-3 is recorded in Table V.

TABLE V  
*Fungal Spectrum of Antibiotic 136*

Organism <sup>1</sup>	Activity <sup>2</sup>
<i>Monosporium apiospermum</i>	2,000
<i>Microsporum audouinii</i>	2,000
<i>Sporotrichum Schenkii</i>	4,000
<i>Nocardia asteroides</i> No. 653	4,000
<i>Epidermophyton floccosum</i>	8,000
<i>Coccidioides immitis</i> No. 819	12,000
<i>Geotrichum</i> sp.	12,000
<i>Hormodendrum Pedrosoi</i> No. 275	20,000
<i>Phialophora verrucosa</i>	20,000
<i>Candida Albicans</i> No. 906	20,000
<i>Trichophyton rubrum</i>	24,000
<i>Hormodendrum compactum</i>	24,000
<i>Cryptococcus neoformans</i>	80,000

<sup>1</sup> The cultures of fungal pathogens were obtained from Dr. N. F. Conant of Duke University.

<sup>2</sup> Highest dilution at which 1 g. of preparation 137-NB-3 will inhibit the test organism.

### *The Differentiation of Antibiotic 136 from Streptothricin and Streptomycin*

Since Antibiotic 136 appears to be similar in its chemical properties to streptothricin and streptomycin, a comparison was made of the antibacterial spectra of these three antibiotics. The same technique was followed as in the determination of the bacterial spectrum of Antibiotic 136. The comparative spectra are shown in Table VI.

The ratios of the activities of streptomycin and streptothricin to Antibiotic 136 are given in the last two columns of Table VI. These ratios differ by a factor of 16 in the case of streptomycin and by 22 for streptothricin. Were Antibiotic 136 the same as streptomycin or strepto-

thricin, the ratios for the 10 test organisms should be in much closer agreement than they are.

Antibiotic 136 can be further differentiated from streptothricin and streptomycin by a comparison of the activity of these three antibiotics when assayed in agar and in a broth medium. In Table VII are recorded, in terms of  $\gamma$  of streptomycin base, the activity of the three antibiotics as determined by a paper disc-plate assay method with streptomycin as the standard and *B. subtilis* as the test organism (9). Antibiotic 136 and streptothricin were prepared for assay by diluting to a concentration of approximately 4 streptomycin units/ml. Closest correspondence

TABLE VI

*Comparative Spectra of Antibiotic 136, Streptomycin, and Streptothricin*

Test organisms	Antibiotic 136 <sup>1</sup>	Streptomycin base <sup>2</sup>	Streptothricin <sup>3</sup>	Ratio <i>Antibiotic 136</i> Streptomycin	Ratio <i>Antibiotic 136</i> Streptothricin
<i>S. albus</i> 151	160,000	10,000	1,500	16.0	106.4
<i>B. subtilis</i> Ill.	140,000	40,000	6,000	3.5	23.2
<i>S. aureus</i> 209	160,000	30,000	7,500	5.3	21.2
<i>E. coli</i> lab.	17,000	6,000	1,200	2.8	14.0
<i>A. aerogenes</i> 8308	5,000	5,000	1,200	1.0	4.8
<i>P. vulgaris</i> 8427	6,000	2,000	900	3.0	6.4
<i>A. viscosus</i> 9036	60,000	8,500	3,750	7.0	16.0
<i>Ps. aeruginosa</i> 9027	1,400	550	165	2.5	8.4
<i>B. cereus</i> 9139	2,600	3,000	50	0.86	52.0
<i>S. marcescens</i>	8,500	9,000	1,125	0.94	7.2

(The figures in columns 2, 3, and 4 represent the number of ml. of solution in which 1 mg. of the antibiotic will completely inhibit the growth of the test organism.)

<sup>1</sup> Preparation 137-NB-3.

<sup>2</sup> Values for streptomycin base were calculated from activities of a preparation which was assayed against the F.D.A. Standard.

<sup>3</sup> A preparation which assayed at 150 Waksman units/mg.

among the assay curves of the three antibiotics is obtained at this level. The values for the activity of these antibiotics against the same test organism in a liquid medium are taken from Table VI. It will be seen in Table VII that the ratio of the activity of Antibiotic 136 in broth to its activity in agar is 75 times greater than the ratios for streptomycin and streptothricin.

TABLE VII

*The Relative Activities in Agar and Broth Media of Antibiotic 136, Streptomycin and Streptothricin*

Test organism <i>B. subtilis</i>	Antibiotic 136 137-NB-3	Streptomycin base	Streptothricin
Activity in agar in streptomycin units	52 units/mg.	1000 units/mg.	147 units/mg.
Activity in broth in dilution units/g.	140,000,000	40,000,000	6,000,000
Ratio of activity in broth/agar	1:3,000,000	1:40,000	1:40,000

#### *Factors Influencing Antibacterial Activity of Antibiotic 136*

A decrease in pH of the culture medium from 7.8 to 6.0 results in a decrease in the activity of Antibiotic 136 against the test bacterium. When the antibiotic was diluted in agar at pH 7.8, 7.5, 7.0, 6.5, and 6.0 the relative activities against *B. subtilis* were 1.0, 0.5, 0.2, 0.1, and 0.04, respectively. The addition of glucose to the test medium decreases the activity of Antibiotic 136 against *Staph. albus* and *E. coli*. Likewise, an increase in the concentration of sodium chloride in the medium from 0.0 to 0.5% results in a decrease in antibiotic activity against the same two test organisms. Cysteine hydrochloride did not inactivate Antibiotic 136 under the conditions under which streptomycin was inactivated when *B. subtilis* was used as the test organism.

#### *Toxicity of Antibiotic 136*

By intravenous or subcutaneous injection into mice the L.D.50 of Antibiotic 136 is in the order of 0.2 mg./18–24 g. mouse. Death is generally delayed 3–5 days after injection. When administered by stomach tube, Antibiotic 136 is not toxic at levels as high as 2.0 mg. per mouse. In mouse tests in which 0.1 mg. of the antibiotic was injected subcutaneously, or in which 1 mg. of the antibiotic was administered orally by stomach tube, there was no protection against Type I pneumococci.

#### *Production of Antibiotic 136 by Other Isolations of Streptomyces*

Antibiotic 136 or preparations very similar to it have been obtained from eleven other isolations of Streptomyces. These comprise not only

strains of *Streptomyces lavendulae*, similar to the 136B strain, but also strains of a white-spored *Streptomyces* species.

### Fraction B

A comparison of the broth/agar activity ratios of Antibiotic 136 and Fraction B reveals that Fraction B has a lower broth/agar ratio than Antibiotic 136 (Table VIII). This fact suggests that Fraction B is different from Antibiotic 136 and that Fraction B may be streptothricin which has a similarly low broth/agar ratio.

TABLE VIII  
*The Broth/Agar Activity Ratios of Four Preparations  
from S. lavendulae, Strain 136*

Fermentation	Preparation	Carbon fraction	Broth/agar ratio
A	137-NB-3	First	1.00
A	135F-NB-3	Second	0.25
B	144AC-NB-3	First	1.60
B	144AD-NB-3	Second	0.16

*B. subtilis* was the test organism used.

Additional evidence of the similarity between streptothricin and Fraction B is obtained from the analyses of crystalline salts of these antibiotic substances. A recrystallized reineckate and helianthate were prepared from Fraction B and analyzed. The analyses of these salts are given in Table IX where they may be compared with those that have been published for the same salts of streptothricin (11, 12). It will be seen that there is a rather close agreement between the analyses for streptothricin and those for Fraction B.

### DISCUSSION

Of the known antibiotics produced by actinomycetes, Antibiotic 136 appears to be the most closely related to streptomycin and streptothricin. Antibiotic 136, however, can be readily distinguished from streptomycin by its antibacterial spectrum and its antifungal activity. Streptothricin and Antibiotic 136, both of which are produced by strains of *S. lavendulae*, can be differentiated on the basis of their antibacterial spectra, toxicity to mice and their broth/agar activity ratios.



TABLE IX

*Analyses of the Helianthates and Reineckates  
of Fraction B and Streptothricin*

Element	Helianthate		Reineckate	
	Streptothricin (12)	Fraction B	Streptothricin (11)	Fraction B
C	50.36	50.54	24.9	24.79
	50.40	50.43	25.0	25.08
H	5.14	6.34	3.94	4.53
	5.44	6.21	4.3	4.18
N	16.86	15.22	23.8	23.63
	16.84	15.75	24.2	23.51
Cr			10.54	10.96
			10.1	
S		6.89	25.8	26.75
		6.50		

Actinomycetes which produce Antibiotic 136 or antibiotics very closely related to Antibiotic 136, seem to be widely distributed, of frequent occurrence, and easily isolated from the soil. It is to be expected then, that antibiotics which are the same as our Antibiotic 136 or very similar to it may be isolated, studied, and described independently by a number of workers in the field of antibiotics. The disentanglement of the identities of these antibiotics will have to await a more detailed study of them, including Antibiotic 136.

### SUMMARY

Strain No. 136 of *Streptomyces lavendulae* produces an antibiotic, Antibiotic 136, which is similar in its chemical properties to streptothricin but differs from streptothricin in its antibacterial spectrum and its high broth/agar ratio and greater toxicity to mice. The production, extraction, and purification of Antibiotic 136 are described. Spectra are given of its antibacterial and antifungal activity. The production by Strain 136 of an antibiotic resembling streptothricin is described.

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# Interference by Streptomycin with a Metabolic System of *Escherichia coli*<sup>1,2</sup>

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Received May 26, 1947

## INTRODUCTION

One way in which an antibiotic agent might prevent the growth of bacteria is by interfering with the action of an enzyme system involved in an essential metabolic process. Since the general subject of the mode of action of antibiotics and other antibacterial agents has recently been reviewed (1, 2), the present discussion has been limited to streptomycin. Although considerable attention has been given to the effect of streptomycin upon the multiplication (3, 4) and morphology (4) of bacteria, less attention has been given to the effect upon respiratory and other enzymes.

Benham (5) has reported that the endogenous metabolism of *Eberthella typhi* was stimulated by streptomycin. Cohen (6) has suggested that streptomycin may combine with nucleic acid. A recent note by Krampitz, Green and Werkman (7) suggests that streptomycin prevents the oxidation of ribonucleinate. The most extensive study yet presented is that of Henry, Henry, Berkman and Housewright (8), who reported no effect of streptomycin upon a number of enzymes of *Staphylococcus aureus*, including cytochrome-oxidase, catalase, aerobic oxidations and anaerobic fermentations of a number of carbon compounds, dehydrogenase systems, carboxylase, urease, and carbonic anhydrase, but they did observe a partial inhibition of the aerobic oxidation of glycerol. More recently Bernheim and Fitzgerald have observed that streptomycin prevents the oxidation of benzoic acid by *Mycobacterium tuberculosis* 607 (9).

<sup>1</sup> Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.

<sup>2</sup> Partly supported by a grant made by the Commonwealth Fund of New York.

Our own preliminary work with *Escherichia coli* led to somewhat similar results. It was observed that with washed cells the rate of oxidation of glucose, lactate, glycerol, succinate, malate, fumarate, oxaloacetate, and pyruvate, was practically the same in the presence as in the absence of streptomycin. Likewise, streptomycin did not affect the production of carbon dioxide or hydrogen under anaerobic conditions by *E. coli* suspended in nutrient broth. Although the oxygen uptake and carbon dioxide production of *E. coli* in nutrient broth under air or oxygen was diminished by streptomycin, the inhibiting effect was slow in developing and did not become clearly apparent until after 1-2 hours. Moreover, rapid cell multiplication in the absence of streptomycin suggested that the effect was secondary in this case and resulted merely from the fact that the larger population oxidized more substrate.

Attention was next directed to the oxidation of amino acids by washed cells of *E. coli*. It was found that, under proper conditions, streptomycin had a marked effect upon the metabolism of these substances.

### EXPERIMENTAL

The medium found the most satisfactory of several tried for the production of actively metabolizing *E. coli* cells was the following:

Peptone	5 g.
Meat extract	3 g.
Yeast extract	3 g.
Sodium chloride	5 g.
Water	1000 ml.

The sterilized medium was inoculated with *E. coli* and incubated at 37°C. for 36 hours. The cells were then centrifuged off, washed 3 times with 0.9% sodium chloride, and suspended in a volume of 0.9% sodium chloride equal to 1/20 the volume of the original culture. Such cell material could be stored satisfactorily in the refrigerator for 3-4 weeks. After this time, autolysis, indicated by an increased oxygen uptake without added substrate, became troublesome, although little change in the turbidity of the cell suspensions was observable at this stage.

It was found advantageous to dilute the cell suspensions to the concentrations used in the metabolic studies with Krebs-Ringer phosphate solution (10). Also, the addition of a small amount of cocarboxylase tended to diminish differences between different lots of cell material.

Conventional Warburg methods were used throughout. In a typical experiment (cf. Fig. 2) 2 ml. of a suspension of *E. coli* (0.05 mg. bacterial N/ml.) was placed in the main compartment together with 0.1 ml. of 0.01% cocarboxylase solution and 0.2 ml.

of either a solution containing 110  $\gamma$  streptomycin/ml., or 0.9% sodium chloride. In one sidearm was placed 0.2 ml. of 0.1 *M* fumarate (or 0.2 ml. of 0.9% sodium chloride) and in the other 0.2 ml. of 0.2 *M* serine (or 0.9% sodium chloride). The center well contained 0.2 ml. of 10% potassium hydroxide. The flasks were then equilibrated for 10 minutes on the bath, and the fumarate added. Readings were made for 3 hours. The second sidearm was then tipped, and readings continued for another 3 hours.

The turbidity of the cell suspensions at the beginning and at the end of the experiments was determined with a Cenco, Type B-2, photoelectric colorimeter, and the number of cells calculated from a calibration curve obtained by plating (11). The nitrogen content of the cell suspensions was determined immediately after washing by the method of Clark (12).

The methods used in the transamination experiments were those of Lichstein and Cohen (13) and Green *et al.* (14). Transamination by the glutamic-aspartic system was followed by determining the formation or disappearance of oxaloacetate with aniline-citrate reagent. Transamination by the alanine-glutamic system was studied by determining the formation or disappearance of ketoglutarate, or the formation of pyruvate.

The amino acids used in the present experiments were, for the most part, Merck & Co. products. Oxaloacetate, ketoglutarate, and sodium pyruvate were prepared in the conventional manner (10). Solutions of amino acids or other compounds were adjusted to pH 7.3 before being added to the cell suspensions.

## RESULTS AND CONCLUSIONS

Because of the lack of effect of streptomycin upon the oxidation of nitrogen-free carbon compounds by *E. coli*, attention was directed to the oxidation of amino acids. It was found that the rate of oxidation of most amino acids including serine, threonine, alanine, leucine, histidine, arginine, lysine, and glutamate was comparatively small, and was unaffected by streptomycin. In contrast, oxygen uptake in the presence of aspartate was greater in the absence (Fig. 1, Curve A) than in the presence (Curve B) of streptomycin. The effect was rather slow in developing and did not become clearly apparent until after the first hour, even though the streptomycin was added to the cell suspension 3 hours before the aspartate.

Since, according to Quastel and Woolf (15), aspartate is rapidly deaminated by *E. coli* with the formation of fumarate and ammonia, and since qualitative tests showed that considerable ammonia was formed from aspartate under the conditions used in the above experiments, the oxidation of mixtures of fumarate and an ammonium salt was next investigated. It was found that such a mixture behaved in much the same way as aspartate (Fig. 1), the oxidation proceeding

more rapidly without (Curve C) than with streptomycin (Curve D). The oxygen uptake of a mixture of serine and fumarate was also diminished by streptomycin, but here the effect was even slower in developing (Curves E and F).

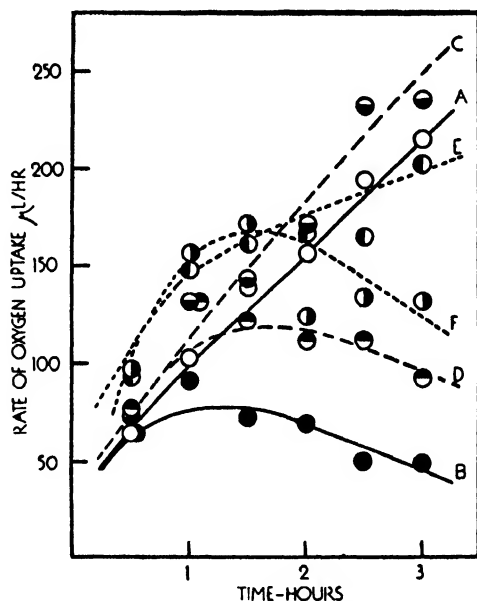


FIG. 1. A: Oxidation of aspartate (0.2 ml., 0.1 *M*) by *E. coli* suspension (2 ml., 0.063 mg. bacterial N/ml.) in Ringer-phosphate solution plus cocarboxylase (0.1 ml., 0.1 mg./ml.). B: Same, but with 8  $\gamma$  streptomycin/ml. C: Oxidation of a mixture of fumarate (0.2 ml., 0.1 *M*) and ammonium sulfate (0.1 ml., 0.2 *N*) by *E. coli* (as above). D: Same, with 8  $\gamma$  streptomycin/ml. E: Oxidation of a mixture of fumarate and DL-serine (0.2 ml., 0.1 *M*) by *E. coli* (as above). F: Same, with 8  $\gamma$  streptomycin/ml.

Upon these observations was based a tentative hypothesis that an oxidation product derived from fumarate was necessary for the utilization of ammonia, or the oxidation of aspartate (formed from fumarate and ammonia by aspartase), or of serine. This suggested carrying out the oxidation of fumarate as a preliminary step, and then adding ammonia or an amino acid. Upon adding an amino acid (*e. g.*, serine) to a suspension of cells of *E. coli* that had been shaken with fumarate for 3 hours, a rapid oxygen uptake ensued (Fig. 2, Curve E) which

exceeded that obtained with fumarate (Curve G) or the amino acid (Curve H) alone, or the sum of these rates. But in the presence of streptomycin, the oxygen uptake was little different from that in the presence of fumarate alone (Curve E). Moreover, the effect was apparent even within one-half hour, and did not seem to be a result of increased cell numbers, since, even after 3 hours, only a 37% increase

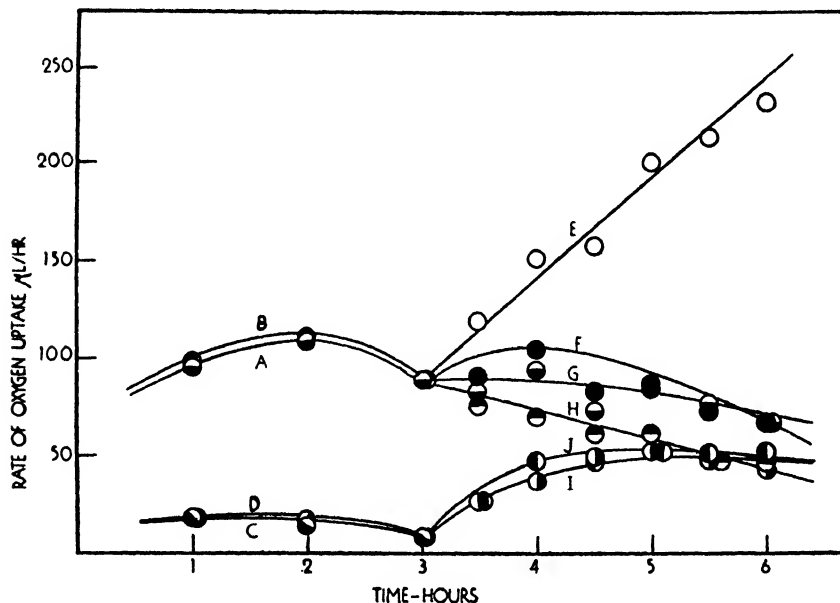


FIG. 2. Effect of a preliminary oxidation of fumarate upon the oxidation of serine by *E. coli*. A: Oxygen uptake by *E. coli* suspension (2.0 ml., 0.051 mg. N/ml.) in Ringer-phosphate solution plus cocarboxylase (0.1 ml., 0.1 mg./ml.), and fumarate (0.2 ml., 0.1 M). B: Same with 8  $\gamma$  streptomycin/ml. C: Endogenous metabolism of cell suspension above. D: Same, with 8  $\gamma$  streptomycin/ml. E: Oxygen uptake after shaking flasks A for three hours, and then adding serine (0.2 ml., 0.2 M). F: Same, with flasks B (containing streptomycin). G and H: Continuation of curves A and B, respectively, after adding 0.2 ml. of 0.9% NaCl at 3 hours. I: Oxygen uptake after adding DL-serine (0.2 ml., 0.2 M) to flasks C after 3 hours shaking. J: Same, with flasks D (containing streptomycin).

in cell numbers had taken place (Table I), while the rate of oxygen consumption in the absence of streptomycin was several times as great as that in the presence of the antibiotic. Although turbidimetric



measurements do not provide a very accurate measurement of viable-cell count, they have been said to give a better measure of total cell substance (16, 17). Their use here is limited to indicating that the change in metabolic rate is far greater than the change in cell numbers. When the streptomycin was added at the same time as the amino acid the oxygen consumption first increased markedly, but then diminished after 0.5-1 hour.

TABLE I  
*Cell Multiplication during Manometric Experiments with Fumarate and Amino Acids (cf. Figs. 2, 4, 5)*

Turbidimetric cell counts at end of experiments

Amino acid	Billions of cells/ml. after treatment with:					
	Fumarate alone		Amino acid alone		Fumarate and amino acid	
	Streptomycin, $\gamma$ /ml.					
	0	8	0	8	0	8
DL-Serine <sup>a</sup>	2.36	2.24	1.62	1.62	2.80	2.04
DL-Alanine <sup>b</sup>	2.90	2.52	2.70	2.28	3.60	2.54
L(-)Leucine <sup>b</sup>			1.84	1.84	2.88	2.24
L(+)Glutamate <sup>b</sup>			1.90	1.90	3.30	2.67
L(-)Aspartate <sup>b</sup>			3.20	2.02	3.46	2.22

<sup>a</sup> At start, 1.90 billion cells, or 0.051 mg. bacterial N/ml.

<sup>b</sup> At start, 2.40 billion cells, or 0.063 mg. bacterial N/ml.

Other amino acids, including alanine, leucine, and glutamic acid behaved in much the same way as serine, as far as the effect of streptomycin was concerned. When added to an untreated cell suspension, their rate of oxidation was small and was not affected by streptomycin. But when these amino acids were added to a cell suspension that had previously been permitted to oxidize fumarate, an immediate increase in oxygen consumption was observed (Fig. 3, Curves B, D, F). This increase was prevented by streptomycin (Curves C, E, G). Serine and alanine (without streptomycin) seemed to undergo a far-reaching degradation, since a rough calculation shows that three or more atoms of oxygen were taken up per molecule of the amino acid, even though the oxidation was incomplete at the end of the experiment. With

leucine, about one atom of oxygen, and with glutamate, about two atoms were utilized.

The addition of aspartate to a suspension of *E. coli* cells that have been permitted to oxidize fumarate for 3 hours resulted in an immediate increase in oxygen uptake in the absence, but not in the presence of streptomycin (Fig. 4, Curves C, D). With aspartate, as previously mentioned, there is probably a side reaction involving deamination and oxidation. This adds the complication that in the controls lacking fumarate and streptomycin (cf. Fig. 1, Curve A) the rate of oxidation increases with time.

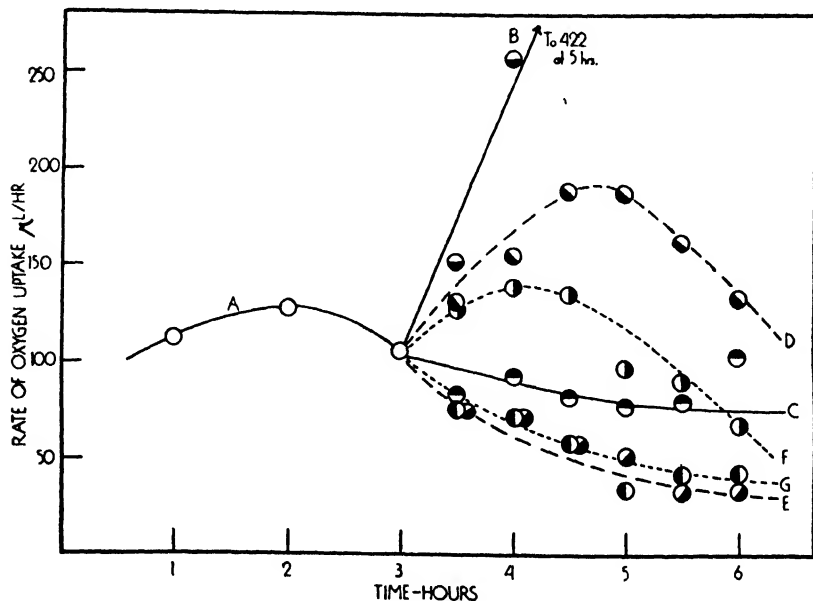


FIG. 3. Effect of a preliminary oxidation of fumarate upon the oxidation of amino acids. A: Oxygen uptake by *E. coli* suspension (2.0 ml., 0.063 mg. N/ml.) in Ringer-phosphate solution plus cocarboxylase (0.1 ml., 0.1 mg./ml.) and fumarate (0.2 ml., 0.1 M). (Curve with 8  $\gamma$ /ml. of streptomycin was identical.) B: Oxygen uptake after adding DL-alanine (0.2 ml., 0.2 M) to flasks like A (no streptomycin) after shaking for three hours. C: Same, but with streptomycin, 8  $\gamma$ /ml. D: Oxygen uptake after adding L(+)-glutamate (0.2 ml., 0.1 M) to flasks like A (no streptomycin) after shaking for three hours. E: Same, but with streptomycin 8 $\gamma$ /ml. F: Oxygen uptake after adding L(-)-leucine (0.2 ml., 0.1 M) to flasks like A (no streptomycin) after shaking for three hours. G: Same, but with streptomycin, 8  $\gamma$ /ml.

Adding an ammonium salt to the usual system (*E. coli* plus oxidized fumarate) led at first to an increased oxygen uptake that fell off rapidly and was not affected by streptomycin (Fig. 4, Curves E and F). This is believed to represent an unrelated phenomenon. Other amines, such as ethylamine, ethanolamine, putrescine, and  $\beta$ -alanine, did not increase the oxygen consumption in this manner.

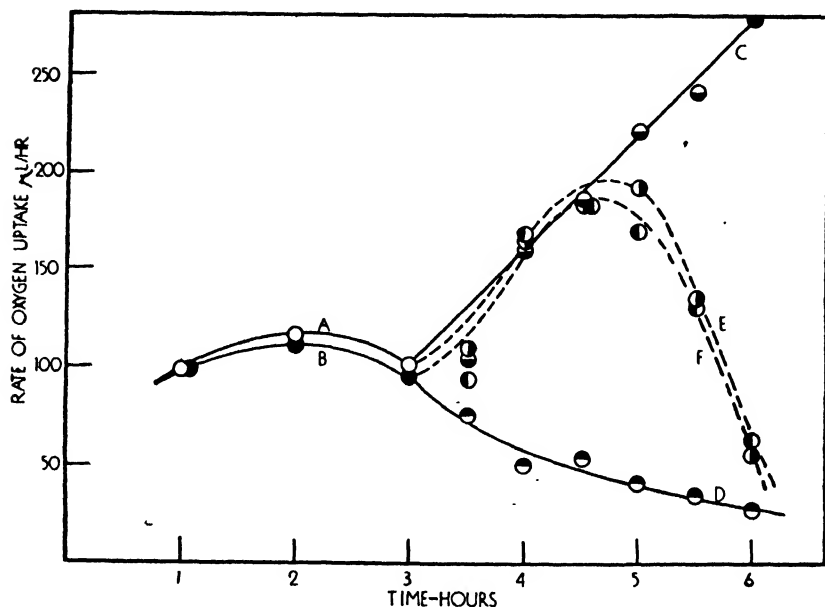


FIG. 4. Effect of a preliminary oxidation of fumarate upon the oxygen uptake upon subsequently adding aspartate or ammonia. A: Oxygen uptake by *E. coli* suspension (2.0 ml., 0.063 mg. N/ml.) in Ringer-phosphate solution plus cocarboxylase (0.1 ml., 0.1 mg./ml.) and fumarate (0.2 ml., 0.1 M). B: Same, with 8  $\gamma$  streptomycin per ml. C: Oxygen uptake after adding L(-)-aspartate (0.2 ml., 0.1 M) to flasks A after shaking for three hours. D: Same, with flasks B (containing streptomycin). E: Oxygen uptake after adding ammonium sulfate (0.2 ml., 0.1 M) to flasks like A after shaking for three hours. F: Same, with flasks like B (containing streptomycin).

The previous experiments were generally carried out at a streptomycin level of 8  $\gamma$ /ml. With smaller concentrations the effect upon the oxidation uptake was less, but was still noticeable at concentrations as

TABLE II

*Effect of Streptomycin upon the Oxidation of Serine by E. coli*

Two ml. of *E. coli* cell suspension (0.051 mg. N/ml.) in Ringer phosphate solution was shaken for 3 hours with (or without) 0.2 ml. of 0.2 *M* fumarate solution and the indicated concentration of streptomycin, and 0.2 ml. of 0.2 *M* DL-serine was then added and the oxygen uptake observed.

Streptomycin	Oxygen uptake/hr./mg. bacterial N, Serine alone	Oxygen uptake/hr./mg. bacterial N, Serine plus fumarate
$\gamma$ /ml.	mm. <sup>3</sup>	mm. <sup>3</sup>
0	587	1450
0.5	627	1720
2.0	460	835
10.0	532	834

small as 0.5  $\gamma$ /ml. (Table II). In these experiments the streptomycin was added to the original cell suspension before the substrates.

These results, considered as a whole, suggested that an unidentified intermediate formed in the course of the oxidation of fumarate by *E. coli* was a necessary part of the amino acid-metabolizing system of this organism, and that the metabolism of amino acids in the presence of this intermediate was prevented by streptomycin. The oxidation of other carbon compounds as a source of the intermediate was also studied (Table III). It was found that the hypothetical intermediate

TABLE III

*Rate of Oxidation of Serine in the Presence of Various Carbon Compounds*

Two ml. of *E. coli* suspension (0.063 mg. N/ml.) in Ringer phosphate solution containing cocarboxylase (0.1 ml., 0.1 mg./ml.) was shaken for 3 hours with 0.2 ml. of 0.1 *M* solution of the compound in the first column, and 0.2 ml. of 0.2 *M* DL-serine was added and the oxygen uptake observed.

	Oxygen uptake/hr./mg. bacterial N,* Streptomycin absent	Oxygen uptake/hr./mg. bacterial N,* Streptomycin, 8 $\gamma$ /ml.
	mm. <sup>3</sup>	mm. <sup>3</sup>
Fumarate	1032	428
Malate	1510	540
Succinate	1670	667
Oxaloacetate	1080	357
Glucose	2090	675
Lactate	1150	572
Pyruvate	198	198
Glycerol	1590	810

\* Average for 3 hours, except for lactate, pyruvate, and glycerol, where the figures represent a 2-hour average.

was also formed from malate, succinate, oxaloacetate, glucose, lactate, and glycerol, but not from pyruvate. The formation of the intermediate from fumarate was greatly facilitated by the presence of phosphate (Table IV). Addition of cocarboxylase was also helpful, while an effect of magnesium was less clearly demonstrable.

Experiments intended to show whether the hypothetical intermediate was associated with the cells or with the suspending medium were also carried out. A suspension of *E. coli* cells was shaken in air with fumarate and cocarboxylase for 3 hours. The cells were then centrifuged and resuspended in Ringer-phosphate solution, and serine was added. It was found that when streptomycin (8  $\gamma$ /ml.) was present throughout

TABLE IV

*Effect of Suspending Medium upon Oxygen Uptake by E. coli  
in the Presence of Fumarate and Serine*

Two ml. of *E. coli* suspension (0.063 mg. N/ml.) was shaken with 0.2 ml. of 0.1 *M* fumarate for 3 hours, and 0.2 ml. of 0.2 *M* DL-serine was then added and oxygen uptake observed.

	Oxygen uptake/hour/mg. bacterial N*	
	Streptomycin absent mm. <sup>3</sup>	Streptomycin 8 $\gamma$ /ml. mm. <sup>3</sup>
Suspended in:		
NaCl (0.9%)	516	303
NaCl (0.8%)		
Na <sub>2</sub> HPO <sub>4</sub> (0.01 <i>M</i> )	714	413
Ringer-phosphate, magnesium omitted, plus 0.01 mg. cocarboxylase	1230	674
Ringer-phosphate plus 0.01 mg. cocarboxylase	1484	365

\* Average for 3 hours.

the experiment, the cells oxidized serine much less rapidly (130 mm.<sup>3</sup> O<sub>2</sub>/hr./mg. N) than cells not subjected to streptomycin (380 mm.<sup>3</sup> O<sub>2</sub>/hr./mg. N). If streptomycin was present only during the oxidation of fumarate, and no more was added to the resuspended cells, the results were much the same. Cells that had been permitted to oxidize fumarate in the absence of streptomycin were able to oxidize serine rapidly, whether or not streptomycin was added along with the serine.

These experiments suggest that the hypothetical intermediate needed for the rapid oxidation of amino acids by *E. coli* is located within the cells. Furthermore, it has not been possible to demonstrate the formation of such a substance in the suspending medium. The exact point at which streptomycin interferes with the metabolic process is at present unknown. It seems unlikely that streptomycin prevents the formation of the intermediate, since the carbon dioxide production, as well as the oxygen consumption, during the oxidation of fumarate, is not affected by streptomycin. It is possible that the antibiotic may combine with the intermediate and prevent its utilization, or may catalyze its decomposition.

Identification of the intermediate must be left for future work. Since it appears to be formed from lactate and glycerol, it may be a  $C_3$  compound. The necessity of phosphate for its formation suggests that it may be a phosphorylated intermediate. The possibility that the intermediate was carbon dioxide was eliminated by a study of the rate of oxidation of serine in Ringer-bicarbonate (10) solution. Oxaloacetate was also found not to increase the rate of oxidation of serine.

Euler, Adler, Günther and Das (18), and more recently Braunstein and Bychkov (19), have suggested that transamination may be an essential part of amino acid oxidation. Since transamination involves substances that might be formed in the course of oxidation of fumarate, such as oxaloacetate and pyruvate, the effect of streptomycin upon transamination by *E. coli* was studied. Rapid transamination was observed between glutamate and oxaloacetate, and between aspartate and ketoglutarate, but the rate of this transamination was not affected by streptomycin at levels as high as 80 units/ml. No transamination could be detected between ketoglutarate and alanine, or between glutamate and pyruvate.

#### ACKNOWLEDGMENT

The author is indebted to Dr. Dean Burk of the National Cancer Institute for a month's patient instruction in Warburg and other manometric methods. A number of the earlier experiments described herein were performed in Dr. Burk's Laboratory. Thanks are likewise due to Professor Selman A. Waksman for encouragement and guidance, and to Miss Dorothy J. Randolph for technical assistance.

#### SUMMARY

1. The oxidation of amino acids by *E. coli* is greatly facilitated by permitting the cells first to act on fumarate or other carbon compounds.

2. The increased ability of such *E. coli* cells to oxidize amino acids is prevented by streptomycin.

3. The oxidation of amino acids by *E. coli* treated in the above manner is facilitated by phosphate, magnesium, and cocarboxylase.

4. It is suggested that an unidentified intermediate is formed during the preliminary oxidation of fumarate (*etc.*), and that this intermediate is involved in the subsequent amino acid oxidation.

5. It is also suggested that the effect of streptomycin upon *E. coli* cells involves, at least as one mechanism, interference with amino acid metabolism.

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# The Premature Death of White Rats Fed Low Levels of Carotenes from Different Sources

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Received June 2, 1947

## INTRODUCTION

Recently it has been shown that the carotene in different foods and feeds is not as well utilized by the rat for storage of vitamin A in the liver as is carotene dissolved in cottonseed oil (7). Likewise, it has been shown that, at low levels of feeding, sufficient for a little growth for short periods of time, the vitamin A potency of the  $\beta$ -carotene equivalent in plant materials appears to be nearly equal to that of  $\beta$ -carotene dissolved in cottonseed oil (8, 10). H. C. Sherman and associates have shown that the vitamin A value of the ration affects the length of life of white rats (11, 12). The quantity of carotene in the ration affects the number of litters, the number of young, the percentage born alive and the live weight of the young at weaning (1). The work here presented compares the relative values of carotene from different sources and vitamin A by their effects on the maximum weight attained by white rats, and the length of their lives. The comparison was made with cod liver oil, carotene in oil, yellow corn, low potency alfalfa and high potency alfalfa leaf meal, included in the ration at the rate of 0.1 and 0.2  $\gamma$   $\beta$ -carotene equivalent/g. of ration fed. These quantities are higher than those used in previous work but not sufficient for any storage of vitamin A in the liver.

## PROCEDURE

At 21 days of age, litters from mothers which had received a diet free of vitamin A potency since birth of their young, were divided into 5 groups of 8 rats each, including 4 males and 4 females. Each group was equalized as to sex, litter mate and weight as nearly as possible. The males and females were placed in separate cages. They were given distilled water and the ration *ad libitum*. Rats were weighed weekly until



natural death, with the exceptions of some of the rats on carotene in oil and on yellow corn. The basal ration was made up of 59% heated corn starch, 22% heated casein, 4% Wesson oil, 1% irradiated yeast, 9% non-irradiated yeast, 4% salt mixture and 1% salt. To the basal ration, the materials to be tested were added, at the rate of 0.1 and 0.2  $\gamma$   $\beta$ -carotene equivalent/g. The high potency alfalfa leaf meal contained about 180 parts carotene/million at the beginning of the experiment, and the low potency alfalfa about 30 parts/million. The materials were tested each month for their vitamin A potency and the quantity added to the basal ration adjusted. Crude carotene extracts of the alfalfas were purified by shaking with a specially prepared magnesium carbonate reagent (9). The rats were kept at about 80°F. in a cooled room in summer, and during other seasons in a house with the temperature controlled by a thermostat.

To ascertain the  $\beta$ -carotene equivalent of the cod liver oil, the parts/million of spectro vitamin A were multiplied by 2.1. This figure was obtained by determining in U. S. P. reference oil, the relation between the International units of vitamin A potency to the parts/million of spectro vitamin A as determined by the spectrograph (3, 6). Then, by using the standard of 0.6  $\gamma$  carotene as equal to one International unit of vitamin A, the  $\beta$ -carotene equivalent of the oil was determined. The potency of yellow corn was ascertained by the method of Kemmerer and Fraps (5). The carotene for solution in oil was prepared by dissolving commercial carotene in chloroform, precipitating with methanol, filtering off, and then drying the precipitate *in vacuo*. A weighed amount of the purified carotene was then dissolved in a very small amount of chloroform and made up to volume with cottonseed oil (4).

## RESULTS

Results of the three experiments are given in Table I. When carotene was supplied at a level of 0.1  $\gamma$   $\beta$ -carotene equivalent/g. of ration, the rats in the groups receiving carotene in oil and in yellow corn reached the greatest maximum weight and also lived longer than the rats receiving the alfalfas or the cod liver oil. The rats receiving carotene in oil lived an average of 277 days and reached a maximum weight of 261 g. Those receiving yellow corn lived 307 days with a maximum average weight of 252 g. With rats receiving the two alfalfas or cod liver oil the length of life averaged 209, 221 and 203 days with maximum weights of 216, 224 and 237 g. Similar results were secured in the two experiments in which the rats received 0.2  $\gamma$  of carotene/g. of ration; the rats on carotene in oil or yellow corn averaged over 644 or 593 days of life, while those on the two alfalfas or cod liver oil averaged 527, 430 or 492 days, respectively. The average of the maximum weights reached were only slightly higher for the rats receiving carotene in oil or yellow corn than for the others.

The low value of cod liver oil may have been partly due to destruction of vitamin A in the mixture. Fraps and Kemmerer (2) have shown

TABLE I

*Length of Life and Maximum Weight of Rats Fed Vitamin A  
and Carotene from Different Sources*

	Alfalfa high potency	Alfalfa low potency	Carotene in oil	Yellow corn	Cod liver oil
Fed 0.1 $\gamma$ carotene/g.					
Length of life in days, females	226	196	276	249	183
	193	199	285	306	159
	185	180	254	264	196
	220	145	295	279	212
	—	—	—	—	—
Average, females	206	180	278	275	188
males	228	252	303	349	196
	190	232	265	346	219
	240	287	303	328	237
	185	273	276	335	220
	—	—	—	—	—
Average, males	211	261	287	339	218
Average, females and males	209	221	277	307	203
Maximum weight in g., males	225	270	316	303	294
females	177	178	206	200	180
Average, females and males	216	224	261	252	237
Fed 0.2 $\gamma$ carotene/g.					
Length of life in days, females	755	399	700	581	495
	553	426	447	622	414
	476	355	698*	698*	438
	621	549	698*	613	418
	—	—	—	—	—
Average, females	601	432	636	629	441
males	432	305	700	503	516
	489	635	523	648	550
	271	479	696*	374	490
	621	294	696*	698*	615
	—	—	—	—	—
Average, males	453	428	653	555	543
Average, females and males	527	430	644	592†	492
Maximum weight in gs., males	369	360	376	355	335
females	218	215	232	230	226
Average, females and males	294	288	304	293	281

\* After 698 days the experiment was discontinued with 6 of the 8 rats alive on carotene in oil and 2 in the yellow corn.

† After 478 days, the experiment was discontinued with 6 of the 8 rats alive on carotene in oil and 7 on the yellow corn.

TABLE I (Continued)

	Alfalfa high potency	Alfalfa low potency	Carotene in oil	Yellow corn	Cod liver oil
Fed 0.2 $\gamma$ carotene.					
Length of life in days, females	323	291	281	483	236
	423	573	483	471	445
	440	289	471†	461†	403
	358	292	463†	463†	275
<hr/>					
Average, females males	386	361	425	470	340
	573	82†	250	483†	408
	552	547	483†	483†	390
	477	556	483†	471†	425
	377	396	471†	300	449
<hr/>					
Average, males	495	500	422	434	418
Average, females and males	441	431	423†	452†	379
Maximum weight in gs., males	278	298	308	296	284
females	183	184	202	207	202
Average, females and males	231	241	255	252	243

† Not included in average.

that vitamin A in cod liver oil, when mixed with feeds, may be less stable than carotene in oil or in alfalfa.

The maximum weights of the rats receiving 0.1  $\gamma$  of carotene/g. of ration averaged 238 g., compared with 272 and 244 g. for those receiving 0.2  $\gamma$ . The higher quantity of carotene had little effect upon the maximum weight attained. The average length of life, however, averaged 245 days for the rats receiving 0.1  $\gamma$  of carotene/g. of ration, compared with over 538 and 430 days for those receiving 0.2  $\gamma$ . Doubling the quantity of carotene more than doubled the length of life of the rats. This confirms work of Sherman and co-workers (11) who reported, among other results, that on a diet of whole wheat and dried whole milk containing vitamin A equivalent to 3, 6 and 12 International units/g., the length of life of the female rats averaged 724, 801 and 830 days, respectively.

#### ACKNOWLEDGMENT

Grateful acknowledgment is made for the assistance of W. W. Meinke.

## SUMMARY

Rats fed on rations containing 0.1 and 0.2 part/million of  $\beta$ -carotene equivalent lived longer when the sources were carotene in oil or yellow corn than when they were fed cod liver oil or alfalfa of low or high potency in carotene. Rats fed 0.2 part/million of carotene in the ration lived more than twice as long as those receiving 0.1 part/million. The maximum weights attained were only slightly greater when the ration contained 0.2 part/million than when it contained 0.1 part/million.

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# **The Influence of Dicumarol Treatment of Rabbits, Rats and Dogs on the Thromboplastic Activity of Their Brain Suspensions**

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Received June 9, 1947

## **INTRODUCTION**

It has been reported by Bose (1) that treatment of rabbits with dicumarol causes a decrease in the thromboplastic activity of their brain tissues. This was demonstrated by preparing saline suspensions of the acetone-dried brains of dicumarol-treated and normal animals, and comparing the activity of these suspensions on various dilutions of plasma from normal and treated animals.

Stefanini and Blanchaer (2) have confirmed the observation of Bose. They tested the thromboplastic activity of brain suspensions from dicumarol-treated rabbits on undiluted plasma from humans, rabbits and dogs, using a range of calcium chloride concentrations from 0.0005 *M* to 0.02 *M*. They found that the decreased activity of the brain extracts was more marked when the lower calcium concentrations were used.

At the time the report by Stefanini and Blanchaer was published we had just completed a study of the effect of dicumarol on the thromboplastic activity of suspensions prepared from the brains of rabbits, dogs and rats. Our studies were made using only one calcium chloride concentration, namely 0.025 *M*. The brain suspensions were tested for thromboplastic activity on normal plasma, both undiluted and in 1:6 dilution, and on undiluted plasma from the dicumarol-treated animals. In the case of rabbit brain suspensions there was sufficient prolongation of the prothrombin time, when dicumarol-treated rabbits were compared with normal rabbits, to confirm the

findings of Bose, and of Stefanini and Blanchaer. On the other hand, brain suspensions from the dicumarol-treated dogs and rats showed no difference in thromboplastic activity when compared with brain suspensions from the untreated animals.

### EXPERIMENTAL

The dicumarol treatment was given in the same dosage described by Bose. The animals were given dicumarol for 3 days, in doses of 10 mg./kg. to the rabbits and dogs, and 6 mg. per animal to the rats. The rabbits and rats were given the dicumarol by stomach tube as a tragacanth suspension, while the dogs were given the drug in capsules. On the fourth day the animals were sacrificed by exsanguination under ether anesthesia. In one experiment on rabbits the bleeding was done by cardiac puncture without anesthesia. Blood samples were obtained from each animal and mixed with 1/9 volume of 0.1 *M* sodium oxalate for use as test plasmas.

The brains from these animals were removed immediately after death, freed of the pia mater and adherent blood vessels, and dried with acetone by grinding in a mortar. Suspensions of the dried brains were then prepared as described by Quick (3), the suspensions of the brains from the normal and treated animals being prepared simultaneously in order to eliminate, as far as possible, the effect of variation in experimental technique. These suspensions were then mixed with an equal volume of 0.025 *M* calcium chloride. The activity of the suspensions was determined by adding 0.2 ml. to a mixture of 0.1 ml. of fibrinogen with 0.1 ml. of the plasma being used as a test solution. The clotting time was measured from the time of addition of the suspension. In testing the activity of the suspensions, normal plasmas undiluted and diluted 1:6, and undiluted dicumarol plasmas were used. Three treated and three normal animals were used in each experiment. All tests were completed on the same day that the samples were obtained.

A loss in thromboplastic activity of the suspensions and in prothrombin activity of the plasma was to be expected during the time involved in making the tests. In order to reduce the effect of such changes on the data to a minimum, the suspensions were tested against the various plasmas in a randomized pattern based on a 6 by 6 Latin square (4).

### RESULTS AND DISCUSSION

The results obtained in these experiments are given in Tables I-IV. In the rabbits (Tables I and II), brains from the treated animals showed less activity than those from the normal animals, this being more marked in the experiment with unanesthetized animals. This decrease of activity was of lesser magnitude than that described by Bose. In rats and dogs (Tables III and IV), however, there was no difference in the thromboplastic activity of brains from normal and treated animals.

TABLE I

*The Thromboplastic Activity of Brains from Normal and Dicumarol-Treated Rabbits (Unanesthetized)*

Thromboplastin prepared from the brains of	Rabbit No.	Plasma from rabbits								
		Normal (undiluted)			Normal (diluted 1:6)			Dicumarol-treated (undiluted)		
		Rabbit No.			Rabbit No.			Rabbit No.		
		1	2	3	1	2	3	4	5	6
Normal rabbits	1	9.2	9.0	8.8	21.0	20.3	15.8	20.9	35.5	38.3
	2	8.8	9.0	8.6	20.8	19.0	16.0	20.0	28.6	31.0
	3	7.3	7.3	7.0	19.2	17.6	14.3	14.5	19.3	22.4
Dicumarol-treated rabbits	4	10.3	9.5	9.1	21.4	18.0	15.4	19.2	32.3	32.9
	5	11.2	11.8	11.5	25.6	23.5	20.8	33.1	101.0	99.0
	6	10.8	9.9	10.0	23.4	20.2	17.0	20.0	29.4	30.5

The figures in the body of the table show the prothrombin time in seconds obtained when the thromboplastin suspensions from the animals indicated in Col. 2 were tested against the correspondingly numbered plasmas.

The lack of a change in the thromboplastic activity of the brains of dicumarol-treated rats and dogs confirms the impression of Stefanini and Blanchaer when they believe that the evidence does not warrant conclusions as to the mechanism of dicumarol's interference with blood coagulation. From the data obtained on rats and dogs, no interference with thromboplastic activity is apparent. It seems likely that, in the case of brain suspensions, at least, dicumarol interference with thromboplastic activity has a species variability.

There was a marked variability in the activity of the thromboplastin suspensions prepared from the normal brains. This variability has been discussed previously by Aggeler *et al.* (5) and by Hurn, Barker and Magath (6). The latter authors point out the numerous factors involved in the preparation of the dried brain and its suspension which may have an effect on the potency, even though considerable effort is made to maintain constant conditions of preparation. In these experiments the suspensions were prepared simultaneously under as closely identical conditions as it was possible to achieve. In spite of this, the



**TABLE II**  
*The Thromboplastic Activity of Brains from Normal and Dicumarol-Treated Rabbits (Anesthetized with Ether)*

Thromboplastin prepared from the brains of	Rabbit No.	Plasma from rabbits								
		Normal (undiluted)			Normal (diluted 1:6)			Dicumarol-treated (undiluted)		
		Rabbit No.			Rabbit No.			Rabbit No.		
		7	8	9	7	8	9	10	11	12
Normal rabbits	7	10.5	10.8	10.7	33.6	29.6	31.7	144.	25.1	28.8
	8	9.4	10.0	9.7	27.7	26.1	27.9	62.	20.3	22.8
	9	9.2	9.7	10.2	31.3	27.8	29.6	80.	20.2	24.6
Dicumarol-treated rabbits	10	10.6	11.0	11.1	33.8	31.2	33.2	216.	25.2	30.6
	11	11.0	11.4	10.9	33.3	28.8	32.6	160.	27.5	29.2
	12	10.2	10.3	11.0	31.8	27.5	29.2	174.	22.8	26.7

The figures in the body of the table show the prothrombin time in seconds obtained when the thromboplastin suspensions from the animals indicated in Col. 2 were tested against the correspondingly numbered plasmas.

**TABLE III**  
*The Thromboplastic Activity of Brains from Normal and Dicumarol-Treated Rats (Anesthetized with Ether)*

Thromboplastin prepared from the brains of	Rat No.	Plasma from rats					
		Normal (undiluted)			Normal (diluted 1:6)		
		Rat No.			Rat No.		
		1	2	3	1	2	3
Normal rats	1	12.8	14.5	15.7	21.7	23.0	23.7
	2	11.2	12.9	13.8	18.5	20.2	19.9
	3	12.7	14.3	14.8	19.8	23.2	23.0
Dicumarol-treated rats	4	10.6	12.6	13.4	18.1	19.3	19.0
	5	11.3	13.3	13.8	19.7	20.4	21.3
	6	11.5	13.2	13.9	19.6	20.6	20.4

The figures in the body of the table show the prothrombin time in seconds obtained when the thromboplastin suspensions from the animals indicated in Col. 2 were tested against the correspondingly numbered plasmas.

TABLE IV

*The Thromboplastic Activity of Brains from Normal and Dicumarol-Treated Dogs (Anesthetized with Ether)*

Thromboplastin prepared from the brains of	Dog No.	Plasma from dogs								
		Normal (undiluted)			Normal (diluted 1:6)			Dicumarol-treated (undiluted)		
		Dog No.			Dog No.			Dog No.		
		1	2	3	1	2	3	4	5	6
Normal dogs	1	7.4	7.6	7.6	15.4	13.6	15.7	68	43	44
	2	7.7	7.7	7.7	15.5	13.3	16.0	76	41	45
	3	7.1	7.2	7.6	15.7	13.7	15.8	69	46	50
Dicumarol-treated dogs	4	7.4	7.5	7.4	15.8	13.3	16.0	93	56	66
	5	8.0	7.6	8.0	15.2	13.3	15.6	77	50	51
	6	7.3	7.3	7.8	15.4	13.1	15.6	77	54	55

The figures in the body of the table show the prothrombin time in seconds obtained when the thromboplastin suspensions from the animals indicated in Col. 2 were tested against the correspondingly numbered plasmas.

thromboplastins from normal brains in each group showed a variation in their activity which was as great as 1.9 seconds, in tests against normal plasma. In tests against diluted normal plasma and dicumarol plasma the variation became even greater. These observations again suggest that caution should be used in the interpretation of prothrombin determinations unless the tests are performed with great care and adequate controls are used each time the test is made.

#### ACKNOWLEDGMENT

We wish to acknowledge the technical assistance of Miss Annabel Avery, B.A.

#### SUMMARY

A comparison of the thromboplastic activity of brains from normal and dicumarol-treated rabbits, dogs, and rats showed that the administration of dicumarol does reduce the thromboplastic activity of rabbit brain suspensions, but not those of rats and dogs.

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# The Amylase Inhibitor of Leoti Sorghum <sup>1</sup>

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Received June 13, 1947

## INTRODUCTION

The occurrence of a water-soluble amylase-inhibiting substance in the seed of Leoti and Schrock sorghums was reported by Kneen and Sandstedt (1). In solution, it was found to be resistant to boiling temperature. In a further communication (2) the same authors reported that the inhibitor from sorghum differed markedly from that found in wheat inasmuch as it inhibited all tested amylases except those produced by culturing fungi. Inhibition was pronounced with barley  $\beta$ -amylase as well as with the cereal, bacterial, salivary and pancreatic enzymes of the  $\alpha$  type. An additional difference was that the inhibiting action disappeared upon germination of the sorghum grain as contrasted to retention of the inhibitor in germinated wheat.

The identities of some of the naturally occurring amylase inhibitors have been established. The inhibiting action of ascorbic acid (3, 4) and of copper-ascorbic acid (5) on certain amylases has been established. An inhibition of malt amylases by several plant auxins has been reported by Eyster (6). Turner (7) reports that the ingestion of tryptophan or the addition of tryptophan or indole *in vitro* decreased the action of salivary amylase on starch.

The amylase inhibitor found in wheat has not been completely identified. However, it was found to be a water-soluble protein, precipitable by alcohol, and sensitive to oxidizing and reducing agents (8). In view of Turner's (7) observation, it is significant that tryptophan was postulated as an essential constituent of the wheat inhibitor (9).

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The availability of a purified amylase-inhibitor that would be effective under operative conditions would have obvious applications in cereal investigations. Accordingly, a study of the properties of the sorghum inhibitor was undertaken with results as herein reported.

## EXPERIMENTAL

### *Measurement of Inhibitor*

The method for determining inhibitor activity was based on the retardation of the rate of starch dextrinization. A 20 ml. portion of 1% soluble starch buffered at pH 5.0 with HCl-sodium citrate buffer (0.025 *M* HCl, 0.025 *M* sodium citrate) was the substrate used in all cases. The reaction temperature was 30°C. Dextrinization time is the time in minutes required to reach the "red brown" end point with iodine solution (10). Except when otherwise stated, the enzyme solution was barley malt extract made up with 0.2% CaCl<sub>2</sub> as a stabilizing agent.

The solution containing the inhibitor was mixed with the appropriate amount of enzyme in a 50 ml. Erlenmeyer flask and placed in the water bath at 30°C. The volume was diluted to exactly 10 ml. The enzyme and inhibitor were allowed to remain in contact for 5 minutes before adding 20 ml. of 1% buffered soluble starch. The 5-minute incubation period used in the present work was in conformity with the time used previously (1).

In cases where a counter-inhibitor was being studied, the inhibitor and counter-inhibitor were mixed and allowed to stand for five minutes at 30°C. before adding the enzyme. The inhibitor, counter-inhibitor and enzyme were diluted to a total volume of 10 ml. and allowed to stand for 5 minutes at 30°C. before adding the substrate.

The per cent loss in activity of malt amylase through contact with various amounts of inhibitor and for varying lengths of time was calculated using the procedure described by Kneen and Sandstedt (1). No inhibition would be represented by 0% reduction in malt activity, and complete inhibition would be represented by 100% reduction in malt activity. Reductions in activity of over 95% were difficult to evaluate because of the long reaction period. It was not found possible to achieve 100% reduction of activity by the use of the inhibitor.

### *Occurrence and Distribution of Inhibitor in Sorghum*

Kneen and Sandstedt (1) report the inhibitor in two varieties of glutinous or "waxy" sorghum, Leoti and Schrock. Both "waxy" and "non-waxy" samples showed inhibitor activity to a similar degree. Kalo, Early Kalo, Pink Kafir, Atlas Sorgo, Waxy Kafir, and Waxy Club showed no inhibiting action whatever.

From the spectrographic analysis of the ashes from various sorghum grains and their starches (11) it was noted that, of the 14 sorghums reported, 3 had high copper content in the grain ash. These 3 sorghums

were Leoti, Schrock, and Early Sumac. Accordingly, an analysis of Early Sumac was made. This variety also was found to contain the water-soluble inhibitor. The analysis of various sorghums for inhibitor content is given in Table I.

TABLE I

*Influence of Sorghum Variety on Content of Inhibitor*

Sorghums*	Extract mg.	Dextrinization time min.
Control (no inhibitor)		21.5
Schrock	100	172
Leoti Red	100	120
Leoti Red (Hays, Kans.)	100	43.5
Westland	500	20.5
Atlas	500	20.5
Blackhull Kafir	500	20.5
Early Sumac	200	135

\* Ground whole grain.

In order to determine the localization of the inhibitor in the Leoti kernel, a sample grown at Hays, Kansas, was tempered to 18% moisture and milled on the Allis experimental mill. The various fractions were analyzed for inhibitor content and the results are presented in Table II. The results indicate that the germ and bran contain a

TABLE II

*Inhibitor Content in the Milled Fractions of Leoti Sorghum*

Fraction	Per cent of whole grain	Extract	Dextrinization time
—		mg. Control (no inhibitor)	min. 21.5
Original grain	100	100	43.5
Scourings, hulls	15	200	25.5
Scourings, dust	4	200	172
Bran	5	200	234.5 ✓
Overs 50 GG (germ)	3	200	1440
Overs 70 GG	33	200	91
Overs 70 GG (reground overs 50 GG)	2	200	600
Overs 11XX	31	200	29
Through 11XX (flour)	7	200	26

much higher proportion of inhibitor than the other fractions. The sorghum flour contained practically no inhibitor. In contrast, the inhibitor from wheat is found in the endosperm but not in the bran (1).

The bran fraction of the milled sample was used as the source of inhibitor for the study of properties and mode of action.

### *Preparation and Purification*

Routine procedure for the preparation of inhibitor solutions was by extracting one part of the ground sorghum grains or fractions thereof with 10 parts of distilled water for one hour at 30°C. This 1:10 extract was centrifuged, filtered through a cotton plug, brought to a boil, and then cooled rapidly, according to the previously described procedure (1). Further filtration was unnecessary since the boiling caused no precipitation of solids.

By the addition of 10 ml. of acetone to each 5 ml. of 1:10 sorghum extract a large proportion of the inactive material present in the extract precipitated in the form of a white floc and could be removed by centrifuging. All of the inhibitor remained in solution.

It was found that the same amount of impurities could be precipitated by the addition of 2 ml. of 4.0 *M*  $\text{CaCl}_2$  to each 5 ml. of sorghum extract. This separation of impurities from the inhibitor with calcium chloride was achieved at the natural hydrogen-ion concentration of the Leôti extract, pH 5.6. When the filtrate was made alkaline, pH 10.5, by the addition of 2 ml. of 0.2 *N* NaOH the inhibitor was precipitated in the form of a red brown gel. When evaporated to dryness this gel yielded 8 mg. of solid/ml. of original solution.

### *Mode of Action*

The inhibitor has been found to inhibit all of the amylases studied with the exception of fungal amylase (2). Of those used in the present investigation, the cereal amylases were found to be the most strongly affected, and of the cereal amylases studied the barley malt was inhibited to a greater extent than wheat malt. The data are presented in Table III.

From the data of Table III and from previously published results (2), it is remarkable that the inhibitor affects all tested amylases except those produced by fungal growth. That this was not due to inactivation of the inhibitor by the fungal preparation was shown by determining the activity of a mixture of malt amylase, fungal amylase, and inhibitor. The activity found was the summation of that for the fungal alone and that for the malt plus inhibitor, not the summation of fungal and uninhibited malt amylase.

TABLE III  
*The Influence of Leoti Bran Extract on the Dextrinizing  
 Activity of Various Amylases*

Amylase	Bran extract equivalent	Dextrinization time	
		Amylase	Amylase plus inhibitor
	<i>mg. bran</i>	<i>min.</i>	<i>min.</i>
Barley malt	300 <sup>a</sup>	21	87
Barley malt $\alpha$ -amylase	200	7.3	15.8
Wheat malt	300 <sup>a</sup>	17	21
Mold bran (fungal)	200	18	18
Fungal precipitate <sup>b</sup>	100 <sup>a</sup>	16	17
Bacterial precipitate <sup>b</sup>	200	14.5	39.5

<sup>a</sup> An extract of ground whole grain.

<sup>b</sup> Wallerstein Laboratories.

The "reaction" of the inhibitor with the enzyme is reversible; *i.e.*,  $E + I \rightleftharpoons EI$ . This was shown by the fact that it was possible to precipitate all of the amylase in its active form and all of the inhibitor from a mixture of the two which had stood for as long as 18 hours. The quantities of amylase and inhibitor were such that 90% reduction in amylase activity was obtained in a check run on the inhibitor. The separation of the mixture into "active" inhibitor and active enzyme was accomplished by precipitating the amylase with 66% acetone at pH 5.0. After the amylase was removed by centrifuging, the solution was brought to pH 10.5, at which point all of the inhibitor was precipitated.

The action of the inhibitor on the barley malt amylases requires some time to reach equilibrium. In a study using mixtures of 20 mg. of barley malt and 50 mg. of Leoti bran extract, it was found that most of the inhibition occurs in the first few minutes after mixing. However, the reduction in activity progresses slowly up to approximately 4 hours contact time, when an equilibrium appears to be achieved. These data are presented graphically in Fig. 1, Curve 1.

In a further investigation of the time-inhibition relationship, purified barley malt  $\alpha$ -amylase (200 mg.) and a high level of inhibitor (400 mg. of bran) were used. It is apparent that true equilibrium was not achieved even after 10 hours of standing under conditions otherwise favorable to retention of  $\alpha$ -amylase activity. The results are given



graphically in Fig. 1, Curve 2, where per cent reduction in amylase activity is plotted against contact time of inhibitor plus enzyme. Some 80% reduction in activity occurred during the first few minutes of standing and this was increased to almost 95% reduction after 10 hours of contact.

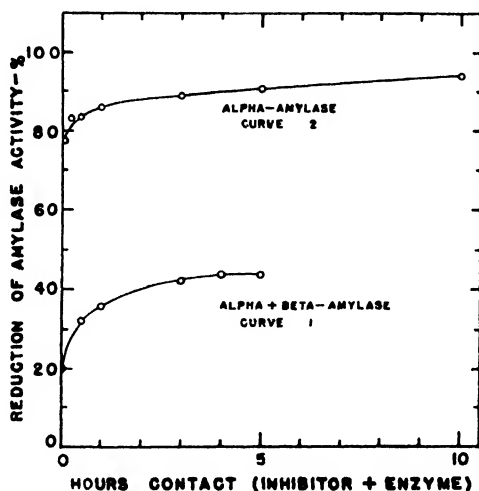


FIG. 1. Relationship of reduction in activity of barley malt and barley malt  $\alpha$ -amylase to the time of contact with the inhibitor.

20 mg. barley malt + 50 mg. Leoti bran extract (Curve 1).

200 mg. barley malt  $\alpha$ -amylase + 400 mg. Leoti bran extract (Curve 2).

#### *Relationship of Inhibitor Concentration to Reduction of Amylase Activity*

For this study various amounts of a 1:10 distilled water extract of Leoti bran were added to uniform aliquots of malt  $\alpha$ -amylase solution. The volumes were brought to a uniform 10 ml. and exactly 15 minutes after the introduction of the inhibitor a 20 ml. portion of 1% buffered (pH 5.0) soluble starch was added. The reaction was allowed to progress at 30°C. Fig. 2 represents the percent reduction in amylase activity corresponding to the indicated inhibitor concentration.

The curve shown in Fig. 2 indicates that a progressive, essentially linear reduction in amylase activity occurred up to the addition of inhibitor equivalent to 300 mg. of Leoti bran. At this point approximately 75% reduction in amylase activity was effected. Above this

point the loss in activity with increasing amounts of inhibitor became less and less pronounced. It may be supposed that, for the amount of amylase used, the inhibitor from 300 mg. of bran was necessary to establish the enzyme-inhibitor equilibrium. It was found impossible to stop completely amylase activity by any amount of inhibitor; hence the curve approaches asymptotically the level of 100% reduction in activity.

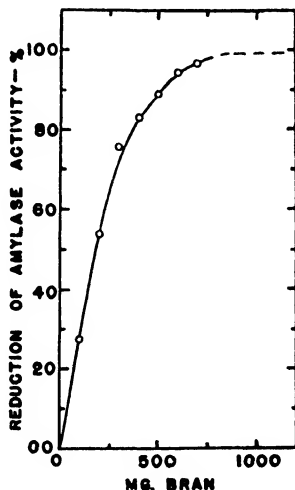


FIG. 2. Relationship of reduction in activity of 200 mg. barley malt  $\alpha$ -amylase to the concentration of inhibitor present.

### *Properties of the Inhibitor*

In contrast to the amylase inhibitor found in wheat (1), the sorghum inhibitor is extremely resistant to inactivation by heat. A 1:10 distilled water extract of the Leoti bran (pH 5.6) was autoclaved at 15 pounds pressure for one hour with no diminution of inhibiting power. This treatment was repeated at a pH of 2.5 and at pH 10.9 with the same lack of inactivation.

The inhibitor was adsorbed from water solution by carbon black (Norit) and could be eluted by 1.0 *N* NaOH. It was thought that this procedure might decolorize the inhibitor solution. However, the removal of the typical red-brown color was accompanied by a complete loss in activity. Under no conditions used could the color be removed

without loss of the inhibitor and it would appear that the inhibitor may be a colored compound. The coloring substance associated with inhibitor activity exhibited a light yellow color in acid solution and a red-brown color in basic solution.

The inhibitor purified by the fractional acetone precipitation procedure gave a positive test for the indole group by the Rosenheim, Adamkiewicz, and bromine tests (12). The nitrogen content (Kjeldahl) was determined to be 2.6%. The formol titration was negative for amino nitrogen and neither nitrous acid nor phenylisocyanate inactivated the inhibitor, thus indicating the absence of essential amino groups.

The inhibitor was stable to storage at room temperature for periods of time as long as two weeks. It was interesting to note that mold growth did not inactivate the inhibitor.

### *Effect of Oxidizing and Reducing Agents*

To test the action of oxidizing and reducing agents on the inhibitor, these agents were treated as counter-inhibitors. Portions of inhibitor extract equivalent to 50 mg. of Leoti bran were used in all cases. Only small amounts of oxidizing and reducing agents could be incorporated because of the effect on the iodine solution used in determining the dextrinization times. One mg. of sodium chlorite had no effect on the inhibitor even when the two were heated to boiling. Two mg. of potassium sulfite, when in contact with the inhibitor at room temperature for 45 minutes, showed no effect. However, when this reducing substance was boiled with the inhibitor, the inhibitory power was markedly decreased. Two mg. of ascorbic acid showed no effect on the inhibitor, even when the mixture was heated to boiling.

It is interesting to point out here that although 0.005 *M* sodium cyanide completely neutralizes the copper-ascorbic acid inhibitor (5) the same concentration of sodium cyanide with two hours contact time had little effect on the inhibitor from Leoti sorghum. Further, the use of 0.005 *M* sodium cyanide as the inhibitor extracting medium did not result in diminution of the inhibitory powers of the Leoti bran extract.

### *Stability Toward Dialysis*

In studying the effect of dialysis on the inhibitor, it was found that dialysis against distilled water for 3 days resulted in an inactive solution. By concentration of the dialyzate, the inhibitor was recovered, thus showing that the molecule was small enough to pass through the pores in the dialyzing membrane. If, however, tap water (pH 7.7) were

used in place of distilled water, the loss of inhibitor was slight even after 3 days of continuous dialysis. Since dialysis against 0.005 *M* dipotassium phosphate (pH 7.9), or 0.03 *M* monopotassium phosphate (pH 4.6), for 3 days also resulted in complete loss of inhibitor, it was shown that passage through the membrane was dependent not on pH but rather on other properties of the dialyzate. Since calcium chloride was found to precipitate the inhibitor from basic solution, it is probable that the calcium ions in tap water prevented the loss of inhibitor during dialysis.

### *Precipitation of the Inhibitor*

Various agents were tried for the precipitation of the inhibitor from aqueous solution. In each case it was found necessary to bring the solution to a reaction of approximately pH 10 before flocculation resulted. To 5 ml. of a 1:10 water extract of Leoti bran was added 2 ml. of 0.2 *N* NaOH. The addition of enough acetone, ethyl alcohol or isopropyl alcohol to bring the solution to 80% solvent by volume was sufficient to precipitate completely the inhibitor in each case. The use of 2 ml. of 4.0 *M* CaCl<sub>2</sub> instead of the solvent was also sufficient to precipitate the inhibitor completely.

Ammonium sulfate was not a satisfactory precipitating agent. Concentrations of this salt up to 70% failed to precipitate more than a small fraction of the inhibitor.

The solubility characteristics may be summed up as follows: On the acid side of neutral the inhibitor is soluble in water, alcohol, acetone or concentrated ammonium sulfate solutions. On the basic side of neutral the inhibitor is precipitated by alcohol, acetone, or calcium ions. Further, it was found to be insoluble in ether.

### *Inactivation of the Inhibitor*

The inhibitor is extremely sensitive to inactivation by water extracts of a variety of biological substances. The data are presented in Table IV.

The only natural inhibitor-free materials tested for counter-inhibitor properties that did not inactivate the inhibitor either in the cold or by heating together were Leoti flour and germinated Leoti. The inhibitor-containing Schrock sorghum likewise did not act as a counter-inhibitor. Most of the natural substances inactivated the inhibitor in the cold; however, it was necessary to heat fungal amylase and malt amylase with the inhibitor in order to destroy the inhibitor.

The addition of peptone to an extract of the inhibitor, prior to the introduction of the amylase, completely inactivated the inhibitor.

TABLE IV  
*The Influence of Various Substances on the  
 Activity of the Inhibitor*

Equivalent weight of inactivating substance		Dextrinization time		
Substance		With inhibitor	Inhibitor plus inactivator	Malt only
	mg.	min.	min.	min.
Wheat bran	200	34 $\frac{3}{4}$	20 $\frac{3}{4}$	21 $\frac{1}{2}$
Westland sorghum	200	34 $\frac{3}{4}$	21	21 $\frac{1}{2}$
Atlas sorghum	200	34 $\frac{3}{4}$	21	21 $\frac{1}{2}$
Blackhull sorghum	200	34 $\frac{3}{4}$	20 $\frac{3}{4}$	21 $\frac{1}{2}$
Mold bran	100	34 $\frac{3}{4}$	21 $\frac{1}{2}$	21 $\frac{1}{2}$
Barley malt	100	100	23	22 $\frac{1}{2}$
Flour, wheat	100	39 $\frac{1}{2}$	22	22 $\frac{1}{2}$
Papain, autoclaved	200	48	22 $\frac{1}{2}$	21 $\frac{1}{2}$
Ficin, autoclaved	200	180	22	22
Starch	200	38 $\frac{1}{2}$	22 $\frac{1}{2}$	22
Soybean meal	100	30 $\frac{1}{4}$	20	21
Corn, yellow dent	100	30 $\frac{1}{4}$	21 $\frac{1}{2}$	21
Flax, Linota	100	30 $\frac{1}{4}$	20	21
Rye, Balbo	100	30 $\frac{1}{4}$	20	21
Oats, Fulton	100	30 $\frac{1}{4}$	21 $\frac{1}{2}$	21
Germinated Leoti	100	34 $\frac{3}{4}$	34	21 $\frac{1}{2}$
Leoti flour	100	29 $\frac{1}{2}$	29 $\frac{1}{4}$	21 $\frac{1}{2}$
Shrock sorghum	10	34 $\frac{3}{4}$	53 $\frac{1}{2}$ <sup>a</sup>	21 $\frac{1}{2}$

<sup>a</sup> Shrock alone gave 29 min.

Peptone had the same effect with extracts of Leoti, Schrock, or Early Sumac sorghums or with a basic solution of kaffiroic acid.

It is of interest to note that a sample of Leoti grain that had been germinated for 3 days at 30°C. did not have any counter-inhibitory action. The finding of Kneen and Sandstedt (2) that germination caused complete loss of inhibiting power was confirmed. During germination, then, the inhibiting substance apparently is destroyed or used up rather than neutralized by production of a counter-inhibitor.

#### *Kaffiroic Acid as an Amylase Inhibitor<sup>3</sup>*

A pigmented high molecular weight acid has been extracted from the bran of kafir sorghum by Woods and Colver (13). They designated

<sup>3</sup> We are indebted to Dr. L. L. Woods, St. Augustine's College, Raleigh, N. C., who made the kaffiroic acid and to Dr. C. W. Colver, Chemistry Dept., Kansas State College, who supplied the sample used in this study.

the compound "kafiroic acid," and described it as being "rust-brown" in color with an "approximate empirical formula of  $C_{18}H_{30}N_2O_6$ ." It was considered of interest to investigate its amylase-inhibiting properties. Due to the insolubility of the acid in water it was necessary to dissolve it in 95% ethyl alcohol for the determination of its inhibitory powers. (The same amount of alcohol was added to the control run without inhibitor.)

As shown in Table V, kafiroic acid did have an inhibitory effect on the amylolytic action of malt extract. However, the differences in properties, *e.g.*, solubilities, between kafiroic acid and the inhibitor from Leoti sorghum indicate that the two compounds are not identical.

TABLE V  
*Inhibitory Action of Indole,  $\beta$ -Indole-3-Propionic Acid, and  
Kafiroic Acid on Barley Malt Amylase*

Malt	Inhibitor	Inhibitor	pH	Dextrinization time
<i>mg.</i>		<i>mg.</i>		<i>min.</i>
40	—	—	4.7	10½
40	Indole	100	4.7	10½
40	$\beta$ -Indole-3-propionic acid	40	4.7	14¼
20	—	—	5.0	24
20	Kafiroic acid	10	5.0	38

*Tryptophan, Indole, and  $\beta$ -Indole-3-Propionic  
Acid as Amylase Inhibitors*

Much evidence points to the connection of the indole group with inhibitor activity (7, 8, 9). Accordingly, several compounds containing this group were tested for amylase inhibition. It was found necessary in all cases to dissolve the compound in ethyl alcohol before adding it to the malt solution because of the low solubility of these substances in water. The same amount of alcohol was added to the control solution. The data are included in Table V and elaborated for tryptophan in Table VI. Of the compounds tested,  $\beta$ -indole-3-propionic acid was the only one which showed amylase inhibitory powers. Tryptophan in a concentration of 0.01 *M* based on the 30 ml. of dextrinization solution failed to inhibit any of the amylases tested, including samples of salivary amylase from a person with extensive dental caries. Accordingly, though indole may be an essential part of the inhibitor molecule,

TABLE VI  
*Influence of 0.01 M Tryptophan on the Activity  
 of Various Amylases*

Enzyme	DL-Tryptophan mg.	Dextrinization time min.
Barley malt	—	20½
Barley malt	60	20½
Fungal amylase	—	12½
Fungal amylase	60	12½
Salivary amylase <sup>a</sup>	—	16½
Salivary amylase <sup>a</sup>	60	17
Salivary amylase <sup>b</sup>	—	13
Salivary amylase <sup>b</sup>	60	13
Salivary amylase <sup>c</sup>	—	29
Salivary amylase <sup>c</sup>	60	29

<sup>a</sup> From person with no caries.

<sup>b</sup> From person with no caries.

<sup>c</sup> From person with extensive caries.

amylase inhibition is achieved only by compounds with highly specific complexity.

## DISCUSSION

The properties and the results of the chemical tests made on the water-soluble amylase-inhibiting substance found in *Leoti* sorghum indicate that it is an organic acid of relatively high molecular weight. However, it does not appear to be identical with any of the inhibitors previously described in the literature. The inhibitor from wheat endosperm reported by Kneen and Sandstedt (1) is distinguished from the *Leoti* inhibitor by being destroyed during autoclaving, by its inability to pass through a cellophane dialysis membrane, and by its general protein characteristics. The lack of inhibition of the *Leoti* inhibitor by sodium cyanide also differentiates it from copper-ascorbic acid, which was completely neutralized by 0.005 *M* sodium cyanide (5). The inhibitory power of kaffiroic acid from Kafir sorghum is interesting. However, this substance, too, is differentiated from the *Leoti* inhibitor, in part by its insolubility in water.

On the basis of this study, and on the many others in the literature which deal with naturally-occurring inhibitors, it may be reasonably assumed that *nearly* all, if not all, biological materials may contain substances which may be classed as enzyme inhibitors. A consideration of the inhibitor from wheat endosperm, the inhibitor from *Leoti* sor-

ghum, and kafiroid acid from Kafir sorghums reveals that all three appear to contain the indole group as a part of their structures. This fact, together with the inhibitory action of  $\beta$ -indole-3-propionic acid, indicates that the indole group as a structural integer is important in the inhibition of amylases.

The ability of the great variety of biological substances for rendering the Leoti inhibitor inactive appears to nullify any plans for the inhibitor to be used as a research tool in studying the effect of amylase in breadmaking or fermentation reactions. However, this sensitivity to inactivation by biological materials does mitigate against any unfavorable nutritional aspect. It is highly improbable that the inhibitor could remain active after ingestion by animals. In fact, when incorporated with an extract of cow's rumen contents the inhibitor was inactivated very rapidly. If problems were encountered, for example in industrial fermentations, one apparent solution would be to mill the grain, thus removing the inhibitor-containing bran and germ fractions.

The high copper content in the 3 sorghum varieties likewise containing the water-soluble amylase inhibitor is rather striking. However, a concentration of  $\text{Cu}^{++}$  equivalent to 40 times the copper content in the sorghum grain showed no inhibitory powers on malt amylase.



### SUMMARY

The properties and mode of action of the amylase-inhibiting substance obtained from the bran fraction of Leoti sorghum are discussed. The presence of the inhibitor in Early Sumac was also noted. This has not been reported by previous workers.

The inhibitor was precipitated from basic solution by acetone, ethyl alcohol, isopropyl alcohol and calcium chloride, but in all instances it was soluble in acid solutions.

Autoclaving the inhibitor at pH 2.5 or pH 10.9 for 30 minutes at 15 pounds pressure produced no inactivation. Stability to standing in the presence of 1 *N* HCl or NaOH also was indicated.

Sodium sulfite inactivated the inhibitor when heated with it. Sodium chlorite showed no effect.

On dialyzing an inhibitor extract against distilled water the substance passed through the membrane and was recovered. When the dialysis was carried on against running tap water, only partial loss of inhibitor resulted.



The inhibition is reversible. It appears to be the result of physical adsorption and not due to a chemical reaction since peptone and a variety of biological substances inactivate the inhibitor.

The partially purified inhibitor gave a positive test for the indole group and contained 2.6% nitrogen.

The sorghum inhibitor appears to be a relatively high molecular weight organic acid containing the indole group and no amino nitrogen.

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# The Effects of Various Agents Upon the Sensitivity of *Staphylococcus aureus* to Penicillin<sup>1</sup>

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Received June 14, 1947

## INTRODUCTION

The available data relating to the effects of the composition of the medium upon the sensitivity of Gram-positive organisms to penicillin are confined almost exclusively to conditions affecting the rate of bacterial growth, the effects of blood, serum, or other body fluids, or to the simultaneous effects of other bacteriostatic agents. Shwartzman's extensive study (1) of the influence of amino acids and related substances upon penicillin sensitivity was confined to relatively insensitive Gram-negative bacilli. It seemed desirable, therefore, to obtain information concerning the effects, in a chemically defined medium, of individual substances at specific concentrations, such data assuming especial importance in view of their possible implications for the mechanism of penicillin action.

This paper describes the effects of inorganic ions, pH, B vitamins and other growth factors, complex nitrogen sources, and amino acids upon the sensitivity to penicillin of a single strain of *Staphylococcus aureus*.

## EXPERIMENTAL

The experiments were carried out in the following manner: A synthetic medium, adequate to support the growth of the test organism, was prepared. To this was added the material to be tested at the desired concentration. Growth of the organism was then determined in this medium in the presence of a series of penicillin concentrations. From the resulting inhibition curve was calculated the concentration of penicillin

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<sup>1</sup> Presented before Section C at the Boston meeting of the Am. Assoc. Advancement Sci., December, 1946.

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necessary for half-maximum inhibition, this figure being compared with the corresponding one for the unaltered medium. This procedure was adopted to minimize possible non-specific stimulatory or inhibitory effects.

The inorganic salts used were either Baker's Analyzed or Merck Reagent chemicals. The amino acids were either Merck or Eastman products, as were most of the vitamins and growth factors used. Yeast extract, proteose-peptone No. 3, brain-heart infusion and bacto-asparagine were Difco preparations.

Various lots of commercial sodium penicillin were used. The potency of each was ascertained from the manufacturer, and the dry powder was weighed out and dissolved in sterile distilled water to a concentration of 1000-1500 units/ml. These solutions were stored frozen in small volumes until needed, when the entire contents of a vial were used. No variation attributable to differences in source of penicillin was observed. Glass-distilled water was used in all experiments.

Some preliminary data were obtained using the Oxford strain of *S. aureus*. Although this strain grows profusely in ordinary bacteriological media, its growth in synthetic media is sparse and slow. Consequently, the more complete data reported here were obtained using a staphylococcus strain supplied by the hospital bacteriologist. While somewhat less sensitive than the Oxford strain, this organism grows profusely upon a relatively simple medium, and is sufficiently responsive to penicillin for the purposes of the experiment. The composition of the basal medium is given in Table I. All of the components, except glucose,<sup>3</sup> were dissolved to give a solution of

TABLE I  
*Composition of the Basal Medium*

Component	Concentration mg./ml.
Acid-hydrolyzed Casein (Pfanstiehl vitamin-free)	10.0
Glucose	5.0
DL-Tryptophan	0.20
L(-)-Cystine	0.10
Thiamine Hydrochloride	0.0010
Nicotinic Acid	0.0010
FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.010
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.20
NaOAc·2H <sub>2</sub> O	2.0
K <sub>2</sub> HPO <sub>4</sub>	2.0
pH	7.0-7.3

<sup>3</sup> It was observed that when glucose was added to the medium before autoclaving, the responses to penicillin were erratic, and suggested that some inactivation of the antibiotic occurred. This is possibly due to the influence of reaction products of glucose and the amino acids of the medium. Accordingly, the glucose was always sterilized separately. See also Foster and Wilker (2).

2.2 times the desired final concentration. The mixture was then autoclaved and allowed to cool, whereupon sterile 10% glucose was added. The medium was then divided. One portion was diluted with half its volume of sterile distilled water. To the rest was added one-half volume of a sterile solution of the substance being tested at the appropriate concentration. Both media were then inoculated and dispensed in 3.0 ml. volumes. To each set of tubes was then added 1.0 ml. of solutions of penicillin prepared so as to give, when diluted 4-fold, concentrations ranging from 0.00 to 0.020 Oxford units/ml. The cultures were incubated at 37°C. for 18-36 hours. Growth levels were measured turbidimetrically, using a Klett-Summerson photoelectric colorimeter after adding a drop of formalin. The data included in Tables II-VIII represent the average of at least two sets of experiments.

The inocula used in these experiments were prepared from overnight synthetic medium cultures. For experiments with deficient media, the culture was centrifuged, washed twice with saline, and resuspended in saline.

TABLE II  
*Effects of Changes in Concentration of Basal Components*

Component	"Normal" concentration	Concentration studied	$\frac{1}{2}$ Max. inhibition units/ml. $\times 10^3$		Per cent diff.
			"Normal"	Variable	
	<i>mg./ml.</i>	<i>mg./ml.</i>			
Cystine	0.10	0.00	7.2	8.4	+16.7
FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.01	0.00	7.2	8.4	+16.7
NaOAc·2H <sub>2</sub> O	2.0	0.00	7.2	8.3	+15.3
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.20	0.00	7.2	11.9, 10.6 <sup>a</sup>	+47 to +65
		0.10	—	11.5	+42
		1.0	—	7.5	- 7.4
K <sub>2</sub> HPO <sub>4</sub>	2.0	0.00	7.2	9.8, 13.8 <sup>a</sup>	+36 to +92
		0.50	—	10.6	+31
		5.0	—	6.5	-19.7
DL-Tryptophan	0.20	0.00	8.1	— <sup>b</sup>	—
		0.008	—	—	—
		0.080	—	9.1	+12.3
		0.80	—	10.0	+23.5
pH <sup>c</sup>		6.50		7.3	
		6.91		8.1	
		7.30		8.2	

<sup>a</sup> Poor growth in controls.

<sup>b</sup> Medium does not support growth.

<sup>c</sup> pH determined after autoclaving.

TABLE III  
*Effects of Vitamins and Growth Factors*

Compound	Concentration	$\frac{1}{2}$ Max. inhibition units/ml. $\times 10^4$		Per cent diff.
		Basal	Supplemented	
	<i>mg./ml.</i>			
Thiamin·HCl	1.01	7.8	6.6	-15.4
Riboflavin	0.094	6.8 <sup>a</sup>	5.0 <sup>a</sup>	-27.5
	0.10	7.2	7.7	+ 6.9
Ca Pantothenate	0.94	6.8 <sup>a</sup>	10.5 <sup>a</sup>	+35.5
Folic Acid ("Folvite")	1.01	10.4	12.8	+23.0
Nicotinamide	1.26	8.9	5.8	-34.8
Nicotinic Acid	1.25	8.9	7.5	-15.7
Coccarboxylase	1.0	7.7	6.8	-11.7
Pyridoxine·HCl	1.0	12.5 <sup>b</sup>	High and Variable	
Pyridoxal·HCl	0.05	10.3 <sup>b</sup>	11.3 <sup>b</sup>	+ 9.7
Pyridoxamine·2HCl	0.40	12.0 <sup>b</sup>	10.8 <sup>b</sup>	-13.6
Yeast Nucleic Acid (Hoffman-LaRoche)	1.09	10.4	11.3	+ 8.7
Asparagine	1.0	10.4	11.0	+ 5.8
Glutamine	1.0	10.4	11.8	+13.5
p-Aminobenzoic Acid	0.80	9.1	7.5	-17.6

<sup>a</sup> Media filtered before using.

<sup>b</sup> Glucose sterilized with the medium.

TABLE IV  
*Effects of Purines and Miscellaneous Compounds*

Compound	Concentration	$\frac{1}{2}$ Max. inhibition units/ml. $\times 10^4$		Per cent diff.
		Basal	Supplemented	
	<i>mg./ml.</i>			
Uracil	0.74	6.5	6.5	0.0
Xanthine	0.15	6.5	7.5	+15.4
Guanine·HCl	0.12	6.5	5.0	-23.0
Adenine Sulfate	0.78	6.5	6.0 <sup>a</sup>	- 7.7
Choline Chloride	1.0	7.7	8.3	+ 7.8
Creatine Hydrate	1.0	7.7	8.4	+ 9.2

<sup>a</sup> Poor growth in controls.

## RESULTS

A typical standard curve is shown in Fig. 1. In a series of 17 determinations on the unsupplemented medium, the half-maximum inhibitory concentrations of penicillin ranged between 0.0065 and 0.0104 units/ml., the average being  $0.0081 \pm 0.0007$  units/ml. When two

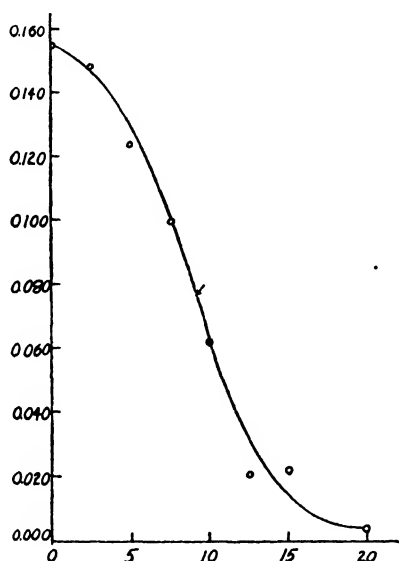


FIG. 1. A typical standard curve. The arrow indicates the point of half-maximum inhibition. Ordinate:  $\log I_0/I$ . Abscissa: concentration of penicillin, units/ml.  $\times 10^3$ .

standard curves were run simultaneously, the deviation was considerably smaller. It is likely, therefore, that differences of more than 20% between the response in supplemented, as compared to unsupplemented media, are significant.

In the first series of experiments, the effects of variations in the concentrations of the constituents of the basal medium were examined. The data are summarized in Table II. Fig. 2 shows the inhibition curves resulting when magnesium- and phosphate-deficient media are used. It may be observed that the most pronounced effects were noted when these two ions were present at quite low concentrations. The possible significance of these results will be discussed subsequently.

The second series of experiments, the results of which are summarized in Table III, included a study of the effects of relatively high concentra-

tions of B vitamins and other growth factors. Some penicillin antagonism was shown by folic acid, calcium pantothenate, and pyridoxine; the latter effect was the most striking, and a more extended study of it

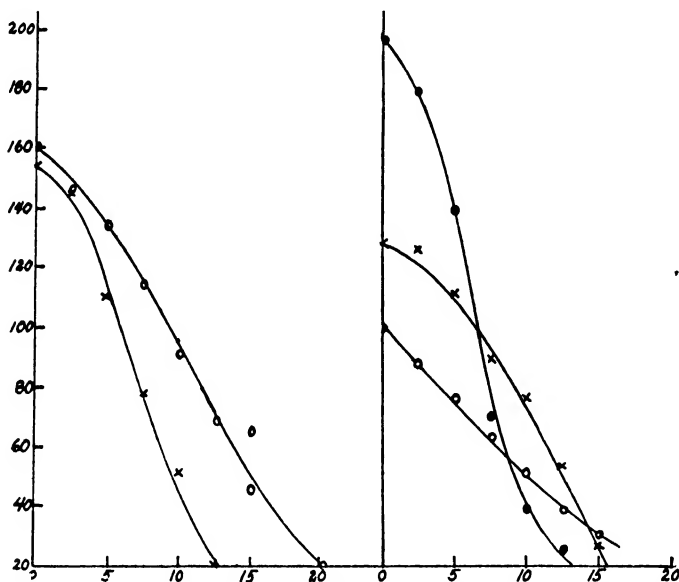


FIG. 2. The effects of variations in  $Mg^{++}$  and  $PO_4^{=}$  concentrations. Left: —○— 0.01%  $MgSO_4 \cdot 7H_2O$ ; —×— 0.10%  $MgSO_4 \cdot 7H_2O$ . Right: —○— 0.00%  $K_2HPO_4$ ; —×— 0.05%  $K_2HPO_4$ ; —●— 0.50%  $K_2HPO_4$ . Ordinates:  $\log I_0/I$ . Abscissae: concentration of penicillin, units/ml.  $\times 10^3$ .

is included later in this report. In agreement with the results of earlier studies (3), nicotinamide, but not nicotinic acid, exerts a synergistic effect. It was observed, however, that the effect of nicotinamide is more pronounced for the Oxford staphylococcus than for the strain used here.

Aside from a slight enhancement observed for guanine hydrochloride, the compounds listed in Table IV were essentially without effect.

The influence of a number of complex substrates upon sensitivity to penicillin is summarized in Table V. It has commonly been stated (4, 5) that conditions favoring rapid growth increase the susceptibilities of various bacterial species to penicillin. It is, therefore, somewhat surprising to find that such an increase is not manifested upon the addition of yeast extract, brain-heart infusion, or proteose peptone,

although these supplements afford much more rapid growth than control experiments. Armour beef extract appears to be synergistic, but the curves were atypical.

TABLE V  
*Effects of Addition of Complex Supplements*

Supplement	Concentration	$\frac{1}{2}$ -Max. inhibition units/ml. $\times 10^3$		Per cent diff.
		Basal	Supplemented	
	<i>Per cent</i>			
Yeast Extract	1.0	8.3	8.7	+ 4.8
Brain-heart Infusion	1.0	8.3	9.5	+14.5
Liver Concentrate (Lederle)	10	8.3	8.2	- 1.2
Proteose-Peptone No. 3	1.0	8.3	9.2	+10.8
Meat Extract (Difco)	1.0	9.1	7.7	-15.4
Meat Extract (Armour)	1.0	9.1	6.6 <sup>a</sup>	-27.3
Human Serum	10	11.25	9.6	-15.0
Rabbit Serum	10	11.25	5.1	-54.5
Fowl Serum	9	11.25	7.7	-31.8

<sup>a</sup> Poor growth in controls.

TABLE VI  
*Effects of Variation of N Source*

N-Source	Concentration	$\frac{1}{2}$ -Max. inhibition units/ml. $\times 10^3$		Per cent diff.
		Basal	Substituted	
	<i>Per cent</i>			
Tryptic Casein Digest	1.0	7.2	6.6	- 8.3
Casamino Acids, Tech.	1.0	11.25	9.7	-14.2
Casamino Acids, Purif.	1.0	11.25	10.1	- 9.8
Amino Acid Mixture	0.97	8.4	6.4	-23.8

In an earlier report (6) it was noted that globulin-free filtrates of human sera exerted a significant synergistic action upon penicillin. The data of Table V reveal similar properties for whole sera of the three species tested, with that of the rabbit being especially potent in this respect.

Table VI indicates the effect of replacing acid-hydrolyzed casein by other nitrogen sources. Apparently, the nature of the process employed



in preparing the casein digest is unimportant with respect to penicillin sensitivity. It would appear, however, that replacing the casein hydrolyzate by a mixture of amino acids results in a definite increase in sensitivity (see Fig. 3).

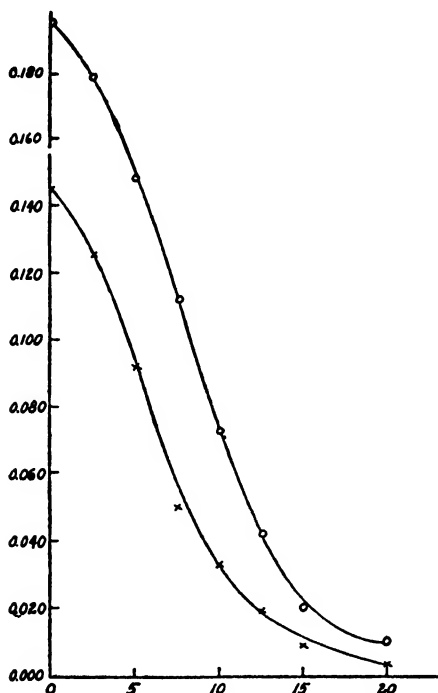


FIG. 3. Comparative effects of casein hydrolyzate (—o—) and an amino acid mixture (—x—). Ordinate:  $\log I_0/I$ . Abscissa: concentration of penicillin, units/ml.  $\times 10^3$ .

The amino acid mixture contained, in addition to cystine and tryptophan, the 15 amino acids listed, with their concentrations, in Table VII. Except for hydroxyproline and isoleucine, the group of amino acids contained all those to be found in casein. The distribution of nitrogen in the amino acid mixture corresponds approximately to that given for casein by Harrow (7). The fact that, in the absence of penicillin, growth in the two media is nearly the same, indicates that the two missing amino acids are of no more than slight nutritional importance.

TABLE VII  
*Composition of Amino Acid Mixture*

Amino acid	Concentration in final medium mg./ml.
Glycine	0.040
DL-Alanine	0.19
DL-Valine	0.84
L-Leucine	1.00
DL-Aspartic Acid	0.40
L-Glutamic Acid	2.25
DL-Serine	0.67
DL-Threonine	0.42
L-Proline	0.83
DL-Methionine	0.42
DL-Phenylalanine	0.42
L-Tyrosine	0.67
L-Arginine Hydrochloride	0.50
L-Lysine Hydrochloride	0.27
L-Histidine Hydrochloride	0.83
Total	9.75

(The above mixture was used in place of the hydrolyzed casein in the basal medium. The other components were present as indicated in Table I.)

TABLE VIII  
*Effects of Variations in Amino Acid Concentration*

Description of medium	Concentration	$\frac{1}{2}$ -Max. inhibition units/ml. $\times 10^3$		Per cent diff.
		Basal <sup>a</sup>	Altered	
	mg./ml.			
Threonine-, methionine-, and aspartic acid-deficient	—	6.4	7.3	+14.1
Glycine varied	0.00	6.5	3.9 <sup>b</sup>	-40
		9.3	3.3 <sup>b</sup>	-65
	0.40	9.3	7.5	-19.3
Threonine omitted	0.00	6.5	6.0	-7.7
Methionine omitted	0.00	6.5	8.4	+30.0
Aspartic acid omitted	0.00	6.5	7.5	+15.4
Valine varied	0.050	—	6.8	
	0.500	—	7.9	
	5.00	—	6.7	

<sup>a</sup> The basal medium contained 0.97% of an amino acid mixture of the approximate composition of casein hydrolyzate.

<sup>b</sup> Uniformly depressed growth.

From the results of the experiments summarized in Fig. 3, it seems probable that casein hydrolyzate contains some factor capable of some antagonism toward penicillin.

Experiments in which single amino acids were omitted from the medium revealed that alanine, valine, leucine, proline, arginine, and histidine were essential for good growth of the organism. Complete inhibition curves in the absence of single amino acids were determined for glycine-, threonine-, methionine-, and aspartic acid-deficient media.

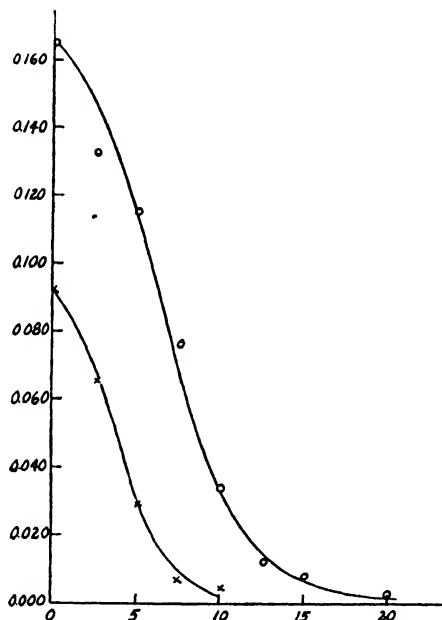


FIG. 4. Inhibition curves for complete (—○—) and glycine-deficient (—×—) amino acid media. Ordinate:  $\log I_0/I$ . Abscissa: concentration of penicillin, units/ml.  $\times 10^3$ .

Significant effects were obtained only when glycine was omitted, the half-maximum inhibitory concentration of penicillin being approximately 50% of the value observed in control experiments (see Table VIII and Fig. 4).

† In view of the presence of a valyl residue in the penicillin molecule (8), it seemed of interest to study the effect of various concentrations of valine upon penicillin activity. As indicated in Table VIII, there was

essentially no change in sensitivity to penicillin over a 100-fold range of valine concentration.

The apparent antagonisms observed for casein hydrolyzate and glycine correspond to the similar effects observed by Schwartzman (1) using Gram-negative organisms. However, the similarities may be only apparent ones, since in one instance the comparisons were made with a simple (Gladstone) medium; in the other, with an amino acid mixture. Shwartzman also observed that aspartic and glutamic acids, cystine, arginine, and histidine were capable of suppressing the antibacterial action of penicillin, and that methionine, methionine sulfoxide, and threonine were capable of reversing their effects. It is possible that the decreased sensitivity apparent in a methionine-deficient medium (Table VIII) may be explained on similar grounds.

#### THE PYRIDOXINE EFFECT

The apparent antagonism of pyridoxine for penicillin (see Table III) was of such striking character that the phenomenon was subjected

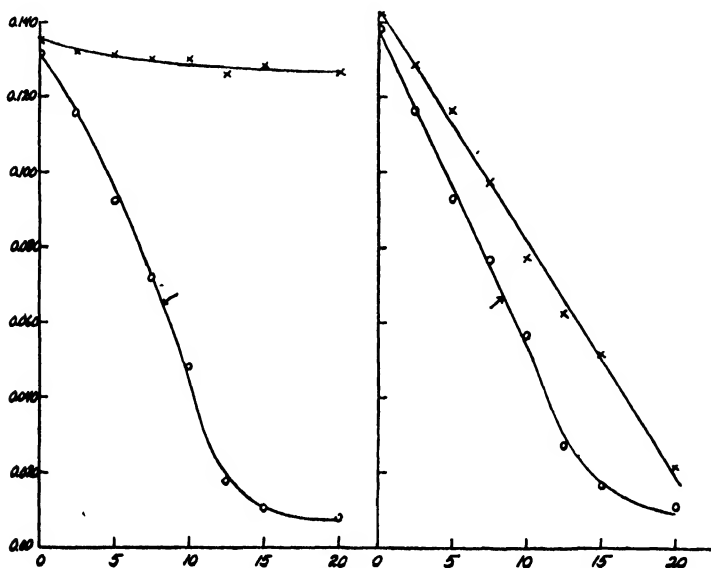


FIG. 5. The inhibitory effects of penicillin alone (—o—) and of penicillin-pyridoxine mixtures (—x—). Left: with previous incubation. Right: without incubation. Medium: proteose peptone; final pyridoxine concentrations 1.25 mg./ml. Ordinates:  $\log I_0/I$ . Abscissae: concentration of penicillin, units/ml.  $\times 10^3$ .

to further study. As noted in Table III, the effects of pyridoxine were quite variable; it seemed reasonable to suppose that the apparent antagonism, as well as the variability of the results, might be due to inactivation of penicillin by reaction with pyridoxine. Appropriate experiments indicated that the antagonistic action is considerably greater if the two substances are incubated together prior to testing. As additional evidence for the correctness of this interpretation may be cited the results of experiments with proteose peptone medium instead of the synthetic mixture. When pyridoxine is added to proteose peptone medium at concentrations of 1.25 mg./ml., the mixture displays a penicillin inhibition curve only slightly flatter than is obtained with the medium alone. If, however, penicillin is incubated with vitamin B<sub>6</sub>, almost complete inactivation occurs, as evidenced by subsequent tests of the antibacterial activity of the mixture in proteose peptone. These experiments are summarized in Fig. 5.

The inactivation of penicillin by pyridoxine appears to be quite specific; in the one experiment performed, both pyridoxal and pyridoxamine were ineffective. Finally, it was apparent that relatively high pyridoxine:penicillin ratios and long reaction times were required for complete inactivation.

### DISCUSSION

In view of the limitations of our knowledge of the functions of specific compounds in bacterial metabolism, it seems pointless to attempt any extended consideration of the data here presented. It is, however, worth while to consider the effect of limited  $Mg^{++}$  concentrations upon penicillin sensitivity in view of two recent reports, dealing, respectively, with the histochemistry of the Gram stain, and of the effect of penicillin upon the utilization of ribonucleic acid.

As is well known, penicillin shares with some other antibiotics the property of being enormously more effective upon Gram-positive bacteria than upon Gram-negative forms. Henry and Stacey (9, 10) have reported a series of studies upon the nature of the dye-retaining constituent. The essential component appears to be the magnesium salt of ribonucleic acid, which was isolated from bile-salt extracts of Gram-positive bacteria. The salt could be replated back onto the Gram-negative cytoskeleton, restoring, in large measure, the Gram-positive character. It was concluded that the Gram-positive constitu-

ent is a nucleoprotein formed by combination of the ribonucleate with a basic protein of the cytoskeleton.

More recently, Krampitz and Werkman (11) have demonstrated that the effect of penicillin upon the metabolic activity of *S. aureus* appears to be wholly upon an endogenous metabolic system which involves ribonucleic acid. The effect is quite specific, and it thus appears that the presence of a ribonucleic acid system is essential for penicillin sensitivity.

The intimate association of  $Mg^{++}$  with ribonucleate, as demonstrated by Henry and Stacey, makes it appear at least possible that the absence of sufficient magnesium affects either the deposition of nucleoprotein or the metabolic system involved in its decomposition. Either of these effects might alter the sensitivity to penicillin, the former conceivably by affecting the permeability of the cell membrane.

It would be interesting to see how the staining characteristics of staphylococci are affected by growth upon Mg-deficient media, and to examine the possible correlation therewith of change in penicillin sensitivity. It might also be profitable to study the effects of the products of ribonuclease hydrolysis of ribonucleic acid upon penicillin activity. Finally, *in vitro* studies of the effect of penicillin upon ribonuclease itself might likewise prove valuable.

### SUMMARY

The effects of inorganic ions, vitamins, growth factors, amino acids, and certain complex mixtures upon the sensitivity of *Staphylococcus aureus* to penicillin have been studied. Reduction in the concentrations of  $Mg^{++}$  or  $PO_4^{=}$  considerably reduces the sensitivity to penicillin, while the absence of glycine exerts an opposite effect. Sensitivity is increased by relatively high concentrations of nicotinamide. Pyridoxine, but not pyridoxal or pyridoxamine, acts as a penicillin antagonist; it has been shown that this effect is due to an *in vitro* inactivation of penicillin. The data have been discussed with a view to their bearing upon the mechanism of penicillin action.

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# Electron Microscope Studies of Dahlem, Rothamsted, and Princeton Samples of Tobacco Mosaic Virus

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Received July 14, 1947

## INTRODUCTION

A number of observations have been made concerning the length of the rod-shaped particles of tobacco mosaic virus as observed in the electron microscope. Kausche, Pfankuch and Ruska (1) in 1939 were the first to report examination of these particles in the electron microscope. They found in a sample of purified tobacco mosaic virus numerous particles having lengths of 300 and 150 m $\mu$ . Melchers *et al.* (2) reported that, for tobacco mosaic virus purified by salting out with ammonium sulphate, the length of the particle occurring most frequently was about 190 m $\mu$ . Stanley and Anderson (3) in 1941 examined centrifugally purified tobacco mosaic virus in the electron microscope and found that the length of the particle occurring most frequently was about 280 m $\mu$ . More recently, Rawlins, Roberts and Utech (4) found in centrifugally purified tobacco mosaic virus a size distribution similar to that found by Stanley and Anderson (3) but having a most common length of about 300 m $\mu$ . Sigurgeirsson and Stanley (5) also found a similar size distribution in freshly expressed juice of plants infected with the virus, and Oster and Stanley (6) have recently demonstrated that electron micrographs of the contents of hair cells from leaves of plants diseased with tobacco mosaic virus showed rod-shaped particles nearly 70% of which were  $280 \pm 20$  m $\mu$  in length.

It has been suggested by Stanley and Anderson (3) and by Schramm and Friedrich-Frekxa (7) that the difference between the length observed by Stanley and Anderson (3) and that observed by Melchers *et al.* (2) may have been due to the fact that the two groups were



examining strains of virus characterized by different lengths. This lack of agreement as to the most probable length, together with the fact that some very short particles appeared in the electron micrographs, led Bawden (8) to suggest that the particles, 280 m $\mu$  in length observed by the American investigators, were aggregates of smaller, possibly spherical, biologically active units. However, it has been shown recently that only particles approximately 280 m $\mu$  or multiples of this length are biologically active (5, 9, 10).

The purpose of the present investigation was to compare, by examination of the contents of hair cells of Turkish tobacco plants infected with the disease, tobacco mosaic virus samples previously studied by Schramm, by Bawden, and by Stanley with respect to the size distribution of particles associated with the disease. Examination of the contents of diseased cells provided a direct observation of the virus as it probably exists in its natural state and eliminated some possible changes in size of the particles brought about by purification procedures.

A comparison was also made of the yield, infectivity, and isoelectric properties of the three samples.

## METHODS

Samples of tobacco mosaic virus were supplied to this laboratory through the kindness of Dr. G. Schramm, formerly of the Kaiser Wilhelm Institute, Dahlem, Germany, and now at the Kaiser Wilhelm Institute in Tübingen, Germany, and of Mr. F. C. Bawden of the Rothamsted Experimental Station, Harpenden, Herts, England. These samples were examined directly in the electron microscope and, together with a sample of the tobacco mosaic virus which has been studied extensively for several years in this laboratory, were applied to separate groups of four-week-old Turkish tobacco plants. All the plants were kept under the same growing conditions.

Twenty-five days after the plants had been infected, the contents of the hair cells of the leaves were examined in the electron microscope by the method of Oster and Stanley (6). This method consists simply of cutting off a few hair cells with a razor blade, gently crushing them on a glass slide with a glass rod and transferring the contents of the hair cells with a drop of distilled water to electron microscope screens mounted on glass slides and covered with a thin film of collodion. After the screens had been allowed to dry, they were dipped into distilled water to remove soluble salts. The metal shadowing technique of Williams and Wyckoff (11) was used to give greater contrast between the virus particles and their background and thus make the images of the particles more distinct. The slides were placed in a high vacuum system (less than  $10^{-6}$  mm. of mercury) and a known amount of gold was evaporated from a hot tungsten filament onto the slides. The slides were placed at an angle of  $15^\circ$  and

were 20 cm. from the source of the gold. From simple geometric considerations (12) the average thickness of the gold on the surface of the samples was estimated to be less than 1  $\mu$ . It was found that the most satisfactory micrographs were obtained when the slides were coated with an amount of gold which gave the slides a pink hue when they were held up to white light.

The samples were examined by means of an RCA Console Model (type EMC-1) electron microscope operating at 30,000 volts and yielding a magnification of 5,800 diameters. The magnification was determined by comparing the sizes of relatively large objects in the electron microscope with those obtained for the same particles in the light microscope. Several determinations were made of the magnification factor, and the value given is probably correct to within 2%. All the micrographs were taken under a low intensity electron beam in order to avoid the beading of the gold observed by Mandle (13). Micrographs were taken of areas of the screens where the particles were far enough apart to be distinct from one another, but where there were sufficient numbers of particles to give statistically significant counts. A micrograph of a sample of purified tobacco mosaic virus in water at a concentration of 0.1 mg. virus per ml. was also taken in order to show the state of virus in a crowded field.

To determine whether the three samples represented different strains, the Turkish tobacco plants infected for twenty-five days were harvested and the virus was centrifugally purified by the method of Stanley (14). The highly purified tobacco mosaic virus samples were obtained after three cycles of differential centrifugation using distilled water as a solvent. The isoelectric points of the samples were determined by a sensitive turbidimetric method (15). The purified samples were diluted to a concentration of  $4 \times 10^{-3}$  mg./ml. in 0.1 *M* phosphate buffer at pH 7 and applied to leaves of *Nicotiana glutinosa* and *Phaseolus vulgaris* in order to determine their specific biological activity (16).

## RESULTS

The Turkish tobacco plants infected with the Dahlem, Rothamsted and Princeton samples of tobacco mosaic virus all showed typical symptoms of the common strain of tobacco mosaic virus. All three groups of the infected plants yielded about 2.2 g. of purified virus per liter of expressed juice. The isoelectric points of the three purified samples in water were found to be at about pH 3.9. The specific biological activities of the three samples were within 10% of one another and, at a concentration of  $4 \times 10^{-3}$  mg. of purified virus/ml., gave an average of 128 lesions per leaf on *Nicotiana glutinosa* and 54 lesions per leaf on *Phaseolus vulgaris*.

Typical electron micrographs of the contents of hair cells from leaves of Turkish tobacco plants diseased with the Dahlem, Rothamsted and Princeton samples of tobacco mosaic virus are shown in Fig. 1. Differences in clarity of the photographs are due to differences in the thick-

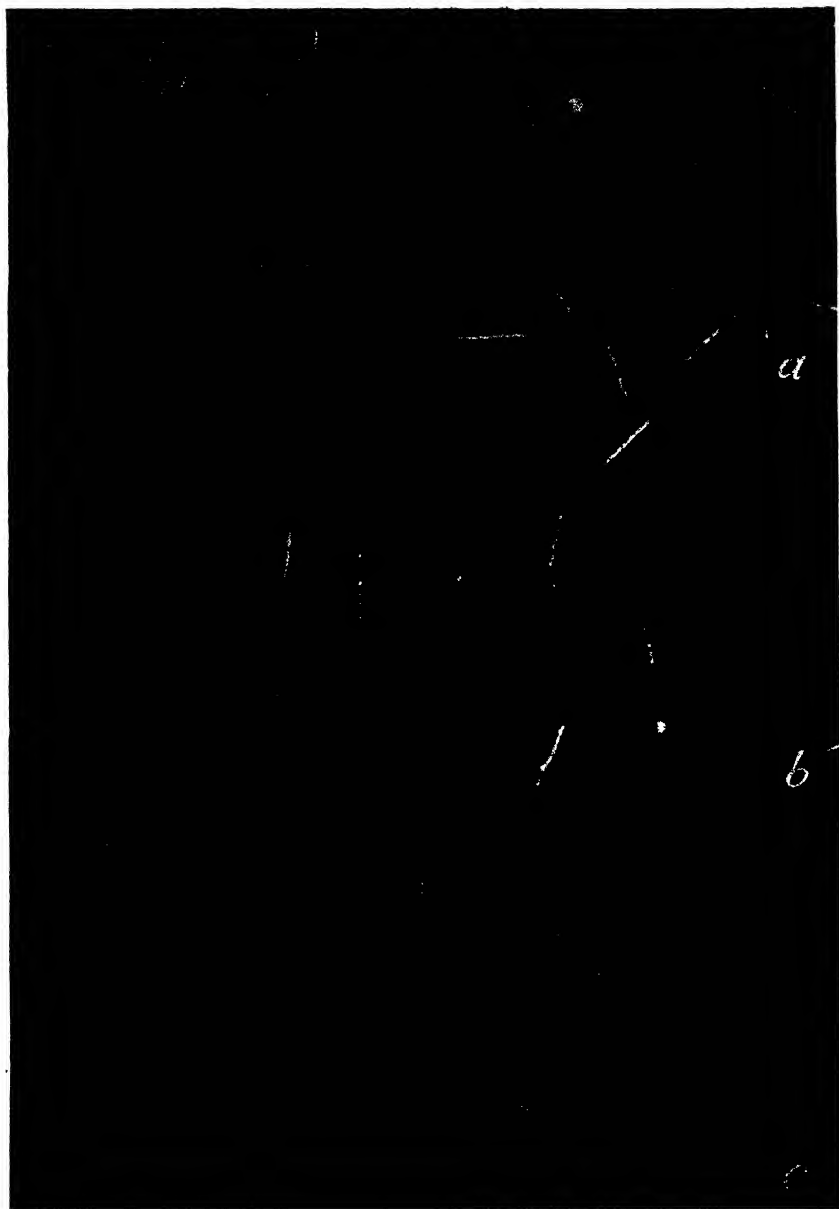


FIG. 1. Typical electron micrographs of the contents of hair cells from leaves of Turkish tobacco plants diseased with the (a) Dahlem, (b) Rothamsted, and (c) Princeton samples of tobacco mosaic virus. Mounts prepared with gold by the shadow casting technique. Magnification = 34,800 $\times$ .

ness of evaporated gold, in the amount of plant material on the mounts, and in the focussing of the electron microscope.

The size distributions of the particles shown in Fig. 1 and in similar micrographs, given in percentages of the total number of particles counted for each sample (about 270 particles), are illustrated in Fig. 2.

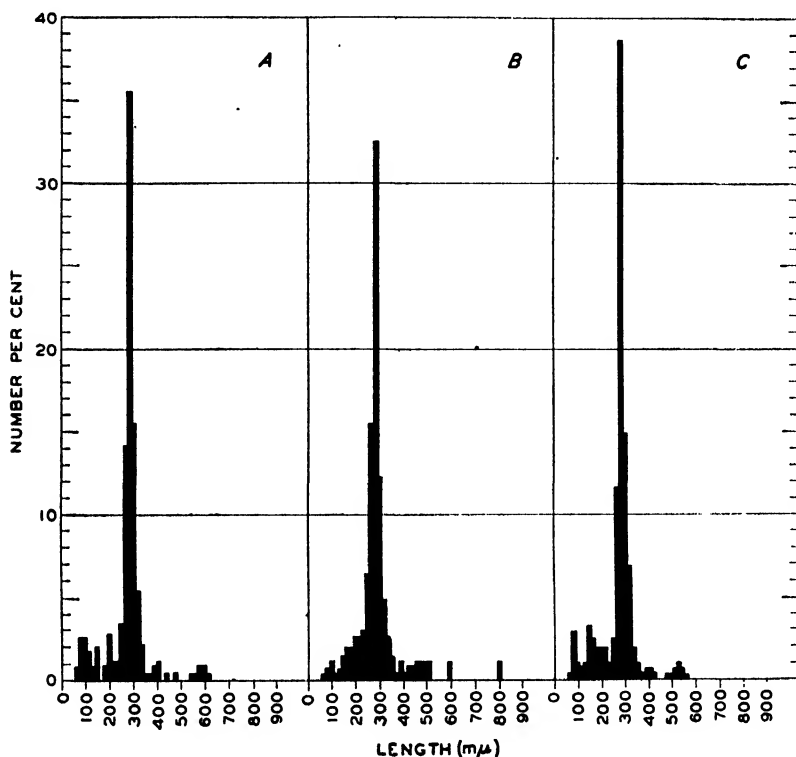


FIG. 2. Size distribution of rod-shaped particles present in the contents of hair cells from leaves of Turkish tobacco plants diseased with the (A) Rothamsted, (B) Dahlem and (C) Princeton samples of tobacco mosaic virus. Distributions represented in percentages of the total number of particles counted (270) for each sample and lengths of particles determined to  $\pm 8.6$  m $\mu$ .

The particles were measured from photographic enlargements (a total enlargement of 29,500 times) of typical electron micrographs and the lengths of all the particles in each picture were measured to the nearest few tenths of a mm. and then distributed according to size in ap-



FIG. 3. Electron micrograph of a concentrated sample of purified tobacco mosaic virus. Mount prepared with gold by the shadow-casting technique. Magnification =  $46,400\times$ .

propriate groups having intervals of 0.5 mm. (equivalent to  $17.2\text{ }\mu\text{m}$ ). As will be discussed shortly, this interval was selected as the smallest one compatible with the accuracy of the measurements. The length under which any given particle was classified was the median of the interval into which it fell and this size could then be considered to differ from the actual size of the particle by no more than  $\pm$  one-half the interval, that is  $\pm 0.25\text{ mm.}$  corresponding to  $\pm 8.6\text{ }\mu\text{m}$ . Thus, a particle measuring  $8.1\text{ mm.}$  in length is placed in the group  $8.0\text{--}8.5\text{ mm.}$  and is considered to possess a length of  $8.25\text{ mm.} \pm 0.25\text{ mm.}$  In Fig. 2 the particle is recorded in the bar at  $280\text{ }\mu\text{m}$  and can be considered to deviate from this length by no more than  $\pm 8.6\text{ }\mu\text{m}$ .

An examination of individual photographs showed that the spread of measurement taken represents the smallest value to which particles of these sizes can be determined practically from gold-shadowed samples in the RCA Console Model electron microscope. This small interval was taken in order to detect possible small variations in the length of the most frequently occurring particle in each of the three samples. There are a number of factors which contribute to the error of measurement. It was found from an examination of electron micrographs of gold sols that the practical resolving power of this microscope is about  $5\text{ }\mu\text{m}$ . For gold-shadowed particles, although there is great clarity of the images, there is an unequal accumulation of gold on the particles which is caused by differences in the orientation of the particles with respect to the direction in which the gold was evaporated. Other errors may be caused by particles not lying in the same plane, by spherical aberration of the electron microscope, by possible changes in the magnification factor of the electron microscope (an error estimated to be less than 5% for this model (17)), by occasional inability of the microscope to give a very sharp focus (see Fig. 1b) and by distortions brought about in the enlarging process, particularly those due to the spherical aberration of the lens of the enlarger and to unequal shrinkage of the photographic paper. Finally, there are normal errors of measuring lengths.

From the size distributions given in Fig. 2 it is seen that the most frequently occurring particle length in the Rothamsted, Dahlem and Princeton samples is  $280 \pm 8.6\text{ }\mu\text{m}$ . Examination of the three original samples before they were applied to the Turkish tobacco plants showed a similar result. If these size distributions are given in intervals of  $40\text{ }\mu\text{m}$ , they are practically identical with that obtained by Oster and

Stanley (6) for the contents of hair cells of plants infected with the Princeton sample in which 800 particles were counted and grouped in intervals of 40  $m\mu$ . They also agree with those found for purified virus by Stanley and Anderson (3) and by Rawlins, Roberts and Utech (4), but do not agree with that found by Melchers *et al.* (2).

The widths of the particles cannot be determined accurately from electron micrographs, such as those of Fig. 1, since the apparent thickness is greater for those particles which have the short axis lying in the direction in which the gold was evaporated. Fig. 3, however, is an electron micrograph of a crowded field of the Princeton sample of tobacco mosaic virus particles (a drop of 0.1 mg. purified virus/ml. distilled water was applied to the electron microscope screens) from which an accurate estimate of the widths of the rods can be made. In the regions where the particles are lying side by side (tactoid regions), the width of such a region divided by the number of particles lying side by side in that region gives a value of 15.2  $m\mu$  for the width of the individual rod-like particles. This value of the width is in excellent agreement with that obtained by Bernal and Fankuchen (18) from X-ray diffraction of dried samples of the Rothamsted tobacco mosaic virus and with that obtained from light scattering studies by Oster, Doty and Zimm (19) on dilute solutions of the Princeton virus.

## DISCUSSION

Since the symptoms of various strains of tobacco mosaic virus on Turkish tobacco plants are usually quite different, the similarity of symptoms shown by the Dahlem, Rothamsted and Princeton samples indicates that all three are probably the same strain of virus. The identity of strain is further suggested by the equal yields of the three samples, since strains examined thus far give considerably smaller yields than does the common tobacco mosaic virus strain (20). The coincidence of the isoelectric points of the three samples is a further indication of their identity, since examination of a number of strains of tobacco mosaic virus showed that, with one or two exceptions, each strain has a characteristic isoelectric point (21).

Fig. 2 shows that the size distributions of the three samples from hair cells of diseased plants are essentially the same, and have maxima at  $280 \pm 8.6 m\mu$ . A similar result was obtained upon direct examination of the original 3 samples. One can, therefore, conclude that, contrary

to earlier suggestions, there is little or no difference in the sizes of the particles of Dahlem, Rothamsted and Princeton tobacco mosaic virus.

It is possible that the shorter particles seen in the micrographs are produced by breakage of the normal-size particles in the drying of the sample on the microscope screen. This explanation, however, does not seem probable since the small particles are distributed uniformly among the larger particles, while, if small particles were produced at the surface of the collodion membrane by drying, one would expect the fragments to be found near one another. Bawden and Pirie (22) and Sigurgeirsson and Stanley (5) have found short particles in the supernatant material of centrifugally purified tobacco mosaic virus. Since normal-size particles can be broken easily by mechanical stresses (10), the short particles may have been produced either in the centrifugation process itself or in the various processes used to prepare the virus, such as fine milling or rapid mechanical stirring. In the present work, however, the extraction of virus did not involve appreciable mechanical agitation of the particles, hence the size distribution is probably the same as that which the virus has in the plant cell. Another possible explanation for the existence of short particles in the hair cell is that they are incompletely formed virus. Preliminary electron microscope studies (23) of the contents of hair cells on plants infected only one day with tobacco mosaic virus seem to indicate that a greater percentage of short particles is found in the early stages of infection than is found at later stages.

The few particles longer than  $280\text{ m}\mu$ , which appear in the micrographs, are probably aggregates of shorter particles, similar to those observed by Sigurgeirsson and Stanley (5) in expressed juice allowed to stand for long periods.

### SUMMARY

Electron microscope studies were made of the contents of hair cells of Turkish tobacco plants infected with the Dahlem, Rothamsted and Princeton samples of tobacco mosaic virus. Size distributions of virus particles from the three samples were found to be essentially the same, with a most common length of  $280 \pm 8.6\text{ m}\mu$ . The significance of shorter and of longer particles also present is discussed. The width of the particles was found to be  $15.2\text{ m}\mu$  by means of the electron microscope. The symptoms on the plants and the yields and isoelectric points of the



purified virus were the same for all three samples. The results indicate that the Dahlem, Rothamsted and Princeton samples are the same strain of tobacco mosaic virus.

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# **The Size of the Particles of Some Strains of Tobacco Mosaic Virus as Shown by the Electron Microscope**

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Received July 14, 1947

## **INTRODUCTION**

Preparations of ordinary tobacco mosaic virus (TMV), which have been obtained under conditions calculated to minimize aggregative and disruptive forces, have been found to contain particles of several sizes, but to be characterized by a preponderance of particles of one specific size. This most frequently occurring entity, which also appears to be the infectious unit of ordinary TMV, can be described as a rod-shaped particle about  $15 \times 280 \text{ m}\mu$  in size (1-4). It is characteristic of TMV obtained from different parts of the world (5) as well as of TMV grown in widely different hosts (6).

Similar, although less extensive, data have been obtained, both by direct and indirect means, for certain distinctive strains of TMV. For example, X-ray evidence indicates that the particles of TMV and of the strains, yellow aucuba (YA) and enation, are  $15.2 \text{ m}\mu$  in width and that the particles of cucumber viruses 3 and 4 (CV3 and CV4), which are commonly considered to be strains of TMV, are about 4% narrower (7). The sedimentation constants in 0.1 *M* phosphate buffer at concentrations of 2.0-3.3 mg./ml. of highly purified preparations of the TMV, YA, green aucuba (GA) Holmes' ribgrass (HR), Holmes' masked (M), J14D1, CV3 and CV4 strains were found to be 188, 191, 192, 187, 180, 185, 186, and 181 *S*, respectively (8). These values were not corrected for solution viscosity, but the data of Lauffer with regard to variation of the sedimentation constant of TMV with concentration (9), indicate that omission of this correction in the concentrations of virus just mentioned would be expected to have a relatively small

effect on the values of the sedimentation constants. It can, therefore, be concluded that highly purified preparations of the strains of TMV enumerated above probably contain particles of essentially the same size and shape. However, in view of the potential significance with regard to virus mutation which the finding of real differences in size might have, it seemed desirable to put this conclusion to test further by examining the virus particles of these strains of TMV in the electron microscope and by making a study of the size distributions of the particles observed. The examination of contents of the hair cells of appropriately diseased plants was selected for this purpose, since this technique eliminates some of the changes in particle size which can accompany purification procedures (10).

### METHODS

The 8 strains of TMV which were studied included the ordinary TMV, M, YA, GA, HR, J14D1, CV3 and CV4 strains. Brief descriptions of the symptoms produced by these strains, as well as the results of a detailed study of the amino acid composition of purified preparations of these strains, were presented recently (11). The first 6 strains were used to infect Turkish tobacco plants and the remaining 2 strains, CV3 and CV4, were used to infect cucumber plants since the latter viruses infect only members of the *cucurbitaceae*. Two to three weeks after inoculation of the plants, the contents of hair cells of infected leaves were used to prepare mounts for the electron microscope as described recently (10). This procedure was repeated more than once for each of the strains so that measurements were made from several electron micrographs in each case and these micrographs represented virus obtained from more than one plant. The lengths of all the measurable particles in each micrograph were recorded in the manner described in the preceding paper (5). A total of 250-300 particles was counted for each strain from photographic enlargements of the micrographs representing a total enlargement of 29,500 times.

A purified preparation of CV4 was employed to prepare mounts with crowded fields of particles in order to permit measurements of the widths of the virus rods as previously described (5). This was considered desirable in view of the X-ray evidence which indicated that the widths of the particles of CV3 and CV4 were slightly less than those of other strains of TMV (7).

### RESULTS AND DISCUSSION

Selected fields illustrating the particles of each strain observed in the electron micrographs are shown in Fig. 1. It will be seen that the particles of the various strains appear very similar in size and shape.

The size distributions of the particles in terms of percentages of the total number of particles measured are given in Fig. 2. In attempting to

evaluate these distributions, the factors contributing to the error of measurement (5) should be considered. In particular, it should be noted that the smallest interval compatible with the accuracy of the

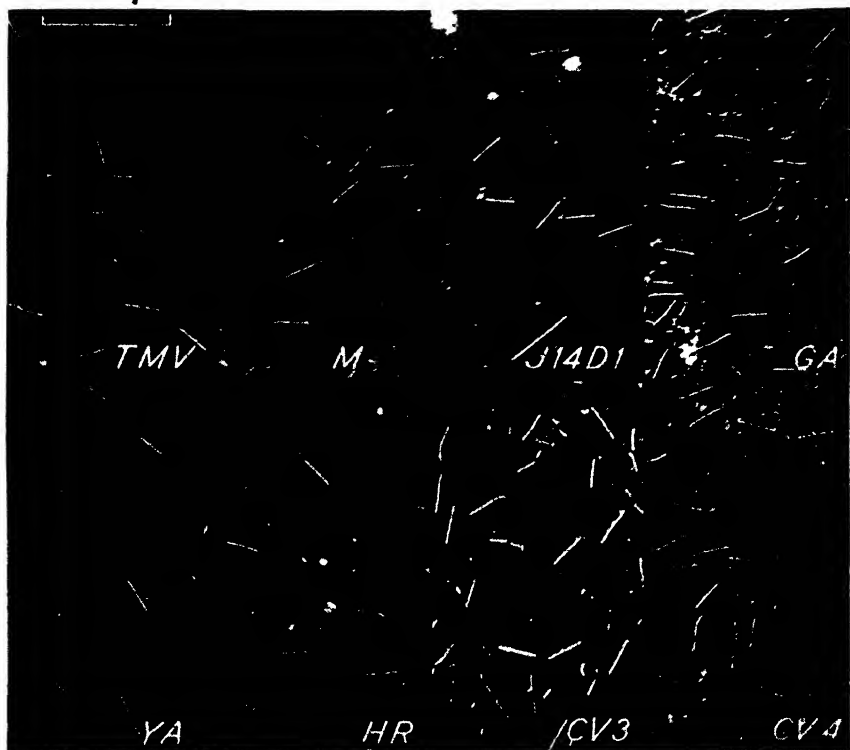


FIG. 1. Electron micrographs of 8 strains of tobacco mosaic virus. TMV, ordinary tobacco mosaic virus; GA, green aucuba; YA, yellow aucuba; HR, Holmes' ribgrass; CV3, cucumber virus 3; CV4, cucumber virus 4. The micrographs are of contents of hair cells from appropriately diseased Turkish tobacco plants except in the cases of CV3 and CV4 which were obtained from hair cells of diseased cucumber plants. Mounts prepared with gold by the shadow casting technique. Line of the micrograph of TMV represents 1  $\mu$ .

measurements was chosen for classification of the particles, in order to detect possible small variations in the length of the most frequently occurring particle in each of the strains. Choice of a larger interval

would, of course, have resulted in a considerable increase in the percentage of particles of the predominant size.

The spreads in particle sizes are largely in the direction of particles smaller than the most common size, which can probably be taken to

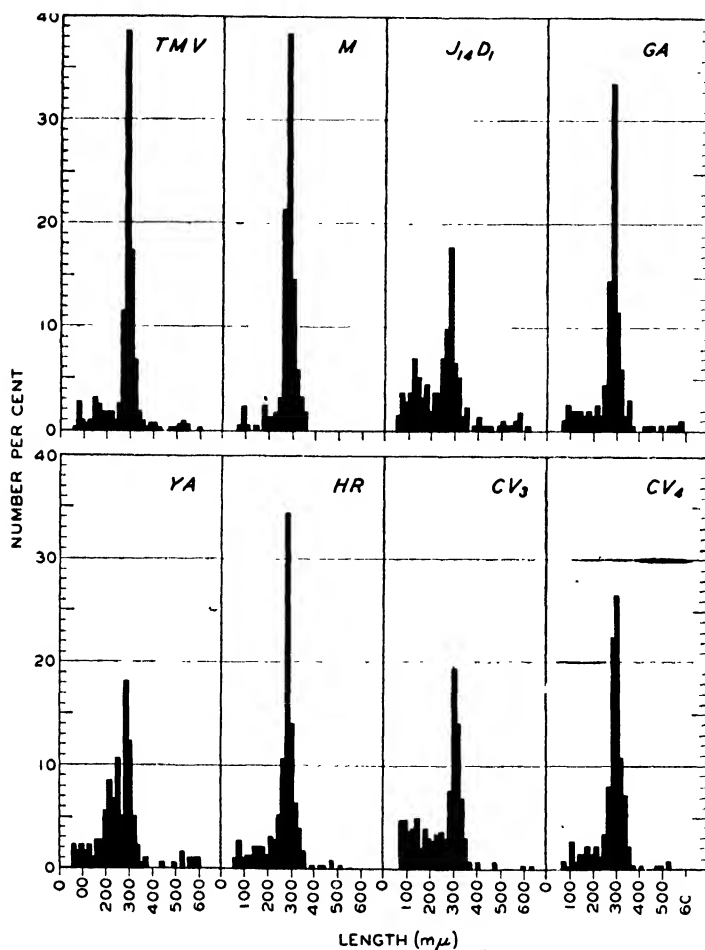


FIG. 2. Size distribution of rod-shaped particles present in the content of hair cells from leaves of Turkish tobacco or cucumber plants diseased, respectively, with 8 strains of tobacco mosaic virus. Distributions represented in percentages of the total number of particles counted (250-300 for each sample). Lengths of the particles determined to  $\pm 8.6$  mμ.

support the argument that the rods of TMV and its strains are ruptured quite easily. The relatively broad spread of particle sizes in some of the distributions shown in Fig. 2 may not be significant, for the distributions in some of the micrographs of J14D1 and YA, for example, showed much greater uniformity of particle size than indicated in the composite analyses in Fig. 2. However the results may be an indication that the strains differ in the ease with which their particles can be ruptured.

As can be seen in Fig. 2, the length of the most frequently occurring particle is  $280 \pm 8.6 \text{ m}\mu$  for all the strains except CV3 and CV4. The latter, in contrast to conclusions drawn earlier from scattered measurements on micrographs of unshadowed preparations (12, 13), appear to be distinctly longer, with a most common length of  $297 \pm 8.6 \text{ m}\mu$ . In addition, the average width of the particles of CV4, obtained by measuring across several particles in a crowded field, was  $13.0 \pm 0.6 \text{ m}\mu$ . This value for the width differs from the X-ray values of  $15.2 \text{ m}\mu$  for TMV and  $14.6 \text{ m}\mu$  for CV3 and CV4 (7), whereas the measurement obtained by this method for TMV (5) coincides exactly with the X-ray value. It thus appears that the particles of CV3 and CV4 differ slightly, but significantly, in size from those of TMV and some of its other strains. This finding is in accordance with general data concerning the relationship of CV3 and CV4 to TMV, for these strains appear to be the most distantly related to TMV of the 7 examined. As mentioned before, CV3 and CV4 have a very restricted host range and possess no host in common with TMV and the other strains; moreover, their amino acid compositions differ in many respects from that of TMV (11). Their relationship to the latter is, therefore, based on their possessing a general size and shape which thus far is unique among viruses, on weak but definite serological cross reactions, on a common resistance to heat and desiccation, and on possession of apparently identical quantities of pentosenucleic acid (12, 14, 15).

The size distributions obtained from electron microscopy agree in 6 instances with the sedimentation results (8) in demonstrating a lack of significant differences among these strains with regard to particle size. The failure to detect a difference between the cucumber viruses and the other strains in the centrifuge can probably be attributed to the smallness of the difference between them, and also to the fact that the difference is of a sort that would compensate partially for itself in the

centrifuge. That is, the rods of CV3 and CV4 appear to be somewhat longer but narrower than those of TMV.

The finding that the particles of TMV and HR are indistinguishable in size is particularly interesting in view of their remarkable divergence in amino acid composition (11). Interest is naturally stimulated by this result to attempt to discover what forces are responsible for building such very similar units from such different amounts and even different kinds of building blocks. It seems evident that the nature of virus reproduction itself is concerned in this problem.

### SUMMARY

Electron microscope studies were made of the contents of hair cells of Turkish tobacco and of cucumber plants infected with 8 distinctive strains of tobacco mosaic virus. Size distributions of the virus particles were similar for these strains and indicated a most common length of  $280 \pm 8.6 \text{ m}\mu$  for 6 of the strains and  $297 \pm 8.6 \text{ m}\mu$  for cucumber viruses 3 and 4. Particles of the latter were found to have a width of  $13.0 \pm 0.6 \text{ m}\mu$  rather than the width of  $15.2 \text{ m}\mu$  considered characteristic of TMV.

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# **The Preparation of Prothrombin by Adsorption on, and Elution from, Aluminum Hydroxide**

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Received July 16, 1947

## **INTRODUCTION**

Quick (1-3) has presented data which he interpreted as showing that prothrombin is composed of two components which he designated as prothrombin A and prothrombin B. One of the procedures which he used for obtaining a plasma containing component A, but not component B, was to treat the plasma with aluminum hydroxide.

We have confirmed the observation that, under the conditions described by Quick, aluminum hydroxide adsorbed component B, furnishing a plasma which had practically no B activity, as shown by its failure to clot when mixed with thromboplastin and calcium chloride, but having a definite A activity as shown by the fact that, on addition of aged plasma, the mixture did clot on addition of thromboplastin and calcium chloride. It seemed possible, therefore, that adsorption with aluminum hydroxide followed by suitable elution would provide a means of preparing a purified preparation of prothrombin B.

Preliminary studies, however, showed that elution of the prothrombin from the aluminum hydroxide with phosphate buffer gave a preparation which contained not just component B, but a complete prothrombin. From these preliminary experiments it appeared probable that a simple method of preparing a fairly pure prothrombin could be developed. A study of various techniques of adsorption and elution led to a procedure by means of which it is possible to prepare a prothrombin solution containing 50% or more of the prothrombin present in the original plasma. Our best preparations have a nitrogen content as low as 4-5 mg./100 ml. It is the purpose of this paper to describe this procedure, together with some of the experiments which



were made to determine the optimal conditions of adsorption and elution.

There have previously been many descriptions of methods for the preparation of prothrombin. Mellanby (4) introduced the procedure of precipitating a crude prothrombin by dilution of plasma with 10 volumes of distilled water followed by isoelectric precipitation at pH 5.3. This has become the initial step in most of the subsequent methods. Seegers and his co-workers (5, 6, 7) introduced the procedure of adsorption of the prothrombin, following resolution, on magnesium hydroxide. It was then removed by conversion of the magnesium hydroxide to soluble magnesium carbonate and further purification obtained in various ways. Milstone (8) follows the initial precipitation by extracting the suspended precipitate with calcium bicarbonate, while Robbins (9) extracts with a phosphate buffer at pH 6.2-6.5.

All these methods involve the use of high dilutions and fairly extreme variations in the pH to which the prothrombin is subjected. In the procedure which we describe here the prothrombin is adsorbed directly from undiluted plasma and is at no time subjected to marked acidity or alkalinity.

## METHODS AND MATERIALS

### *Determination of Prothrombin Activity*

Prothrombin activity was determined by the one stage method (10). This was modified in two ways: (a) since most of the preparations did not contain fibrinogen, 0.1 ml. of fibrinogen, prepared as described by Jaques (11), was added to the plasma or preparation from it; (b) the thromboplastin suspension and calcium chloride solution were mixed in equal proportions, 0.2 ml. of this mixture was added to the prothrombin-fibrinogen mixture, and the prothrombin time measured from this point.

### *Nitrogen Determinations*

Total nitrogen was determined in 1.0 ml. of the eluates by the micro Kjeldahl procedure followed by Nesslerization.

### *Temperature*

Unless otherwise indicated, all experiments were performed at 0°C. The materials used were cooled in a bath of crushed ice and water and kept in such a bath. Centrifuging was done in cups packed with crushed ice, which usually maintained a temperature not above 5°C., even after 60 minutes of centrifuging.

### *Plasma*

Rabbit plasma was used in all these experiments. Blood was collected from the rabbits by cardiac puncture, mixed with 1/9 volume of 0.1 *M* sodium oxalate and the plasma obtained by centrifuging for 1 hour at 2,000 r.p.m.

Preliminary experiments showed that a considerable amount of fibrinogen was adsorbed and later eluted by the procedure we followed. For this reason fibrinogen was removed from the plasma by the addition of 1/10 volume of thrombin<sup>1</sup> followed by removal of the fibrin. This defibrinated plasma was frozen and stored at  $-20^{\circ}\text{C}$ . until used.

### *Aluminum Hydroxide Gel*

A commercial preparation of aluminum hydroxide gel<sup>2</sup> was used as the adsorbent in all this work. It appeared to us that such a preparation would have more consistent properties than any preparation made in small quantities in the laboratory.

### *Oxalated Saline*

This solution was prepared by dissolving 0.92 g. of potassium oxalate in 1,000 ml. of 0.15 *N* sodium chloride (12).

## RESULTS

### *A Satisfactory Procedure for Obtaining an Active Prothrombin Solution*

The procedure which results in the most active prothrombin preparation is as follows: defibrinated plasma is adjusted to pH 8.0, mixed with aluminum hydroxide gel in the ratio of 0.2 ml. of the gel to 5.0 ml. of plasma, and allowed to stand 15 minutes. The mixture is then centrifuged for 1 hour at 2,000 r.p.m., the clear supernatant decanted, the aluminum hydroxide washed 3 times by suspending it in 3 ml. of oxalated saline and the wash discarded after centrifuging for 30 minutes at 2,000 r.p.m. The adsorbed prothrombin is eluted with 0.2 *M* phosphate buffer, pH 8.0. The aluminum hydroxide is broken up with a stirring rod and 5.0 ml. of the buffer added. The tube is stoppered and inverted slowly for 10 min. and then allowed to stand for 15 min. The water-clear supernatant, obtained after centrifuging for 1

<sup>1</sup> Parke, Davis and Company Thrombin Topical—1 ampoule being made up to a volume of 200 ml. with 0.15 *N* sodium chloride. We are indebted to Dr. Eugene C. Loomis for generous supplies of this material.

<sup>2</sup> Wyeth's Amphojel, without flavor. This product conforms to the U.S.P. standards for aluminum hydroxide gel. We are indebted to Dr. Alfred Barol, Director of the Wyeth Institute of Applied Biochemistry, for supplies of this material, and for information regarding its properties.

hour at 2,000 r.p.m., is then adjusted to pH 7.4–7.6 and dialyzed in Visking cellophane tubing, 18/32 inch diameter, against distilled water until free of phosphate. A small amount of precipitate which forms during dialysis is removed by centrifuging.

This procedure was arrived at after studying various modifications of the time and temperature of adsorption, the proportion of aluminum hydroxide used, and the pH and concentration of the buffer used for elution. We consider the best product to be one having a high prothrombin activity while at the same time having a low nitrogen content. Since these criteria are achieved only under rather rigid conditions we wish to show the effect of variation of some of these factors on the nature of the eluate. In these experiments showing the effects of various factors we were studying the optimal conditions for one factor at a time. Since, in most cases, the conditions for the other factors were not optimal, neither the prothrombin activity nor nitrogen content of the eluates are as satisfactory as we now obtain when all conditions are optimal.

*Effect of the pH of the Plasma on the Adsorption of  
Prothrombin by Aluminum Hydroxide*

Five ml. lots of plasma were adjusted to various pH values and then treated with 0.3 ml. of alumina in the usual manner. These data are given in Fig. 1, from which it can be seen that the most active pro-

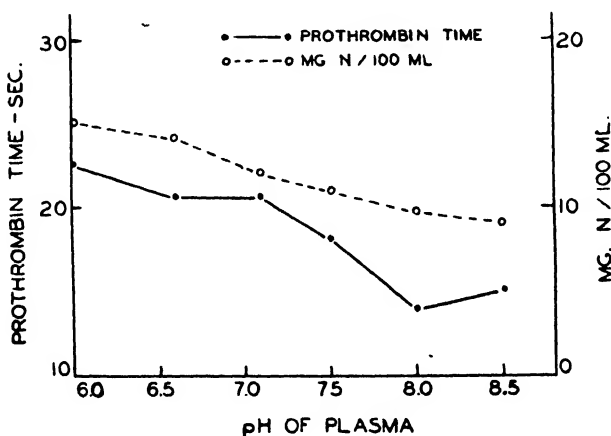


FIG. 1. Effect of the pH of the plasma on the adsorption of prothrombin by aluminum hydroxide.

thrombin preparation was obtained from a plasma which had a pH of 8.0. The amount of nitrogen contained in the eluate decreased steadily from pH 6.0 to pH 8.5.

*Effect of Time and Temperature on the Adsorption of  
Prothrombin by Aluminum Hydroxide*

Five ml. samples of defibrinated plasma were mixed with 0.5 ml. of aluminum hydroxide and treated as follows: one tube was centrifuged immediately; 3 were incubated at 37°C. for 5, 10, and 15 minutes, respectively, and then centrifuged; and 3 were allowed to stand at 0°C. for 5, 10, and 15 minutes, respectively, and then centrifuged. The procedure given above for elution of the prothrombin from the aluminum hydroxide was followed in this experiment. The results, which are given in Table I, indicate that 15 minutes incubation at 0°C. produces

TABLE I  
*Effect of Time and Temperature on the Adsorption of  
Prothrombin by Aluminum Hydroxide*

Conditions	Time of incubation	Prothrombin time	N/100 ml. eluate
	<i>mins.</i>	<i>secs.</i>	<i>mg.</i>
1. Mixed, centrifuged immediately		27.9	24.9
2. Incubated at 37°C.	5	25.5	21.5
	10	31.7	20.6
	15	35.3	19.7
3. Incubated in crushed ice-water mixture	5	20.1	24.2
	10	18.8	24.0
	15	18.4	24.0

5.0 ml. plasma mixed with 0.5 ml. aluminum hydroxide. Prothrombin eluted with 5.0 ml. of 0.2 M phosphate buffer, pH 8.0, by inversion for 10 minutes, then allowing the tubes to stand in an ice bath for 15 minutes.

the most active prothrombin preparation. There is a progressive loss of prothrombin on incubation at 37°C. during the 15-minute period over which the study was made. The nitrogen values are higher than most of those reported in this investigation. This is probably due to the fact

that the adsorption was carried out with 0.5 ml. of aluminum hydroxide. This amount of aluminum hydroxide adsorbs considerable amounts of protein, as can be seen in the section discussing the use of various amounts of aluminum hydroxide.

*The Effect of Varying Amounts of Aluminum Hydroxide on the Prothrombin Activity of the Eluate*

In this study defibrinated plasma was mixed with varying amounts of aluminum hydroxide and the procedure outlined above was followed throughout except that the eluates were dialyzed against cold oxalated

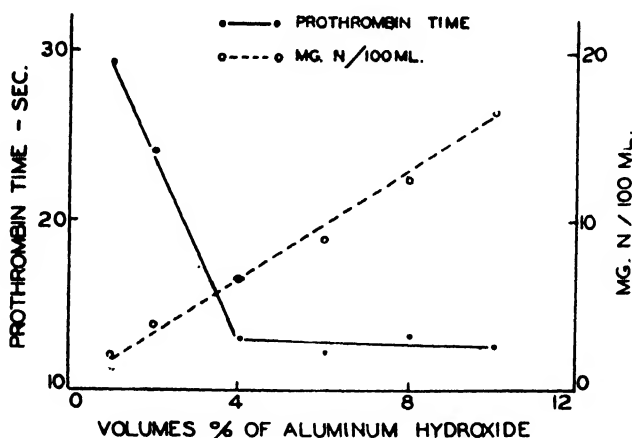


FIG. 2. The effect of varying amounts of aluminum hydroxide on the prothrombin activity of the eluate.

saline. The data obtained are presented in Fig. 2. It is evident that amounts of aluminum hydroxide from 4 to 10% of the plasma volume result in eluates with the same prothrombin activity but with increasing amounts of nitrogen. For this reason we have adopted the use of 0.2 ml. of aluminum hydroxide with 5.0 ml. of plasma in the final procedure for obtaining active prothrombin solutions. However, since the experiment involving the use of 1-4% of aluminum hydroxide was performed at a later time, most of the experiments reported here were carried out using 6% of aluminum hydroxide which give an eluate containing 11-12 mg. of nitrogen/100 ml. of eluate. When the plasma

was absorbed with 4% or more of aluminum hydroxide, the residual plasma gave no clot in 300 seconds after the addition of thromboplastin, calcium chloride, and fibrinogen.

*Effect of the pH of the Buffer on the Elution of Prothrombin from Aluminum Hydroxide*

The effect of the pH of the buffer used in eluting prothrombin from aluminum hydroxide is shown in Fig. 3. The buffers from pH 6.3 to

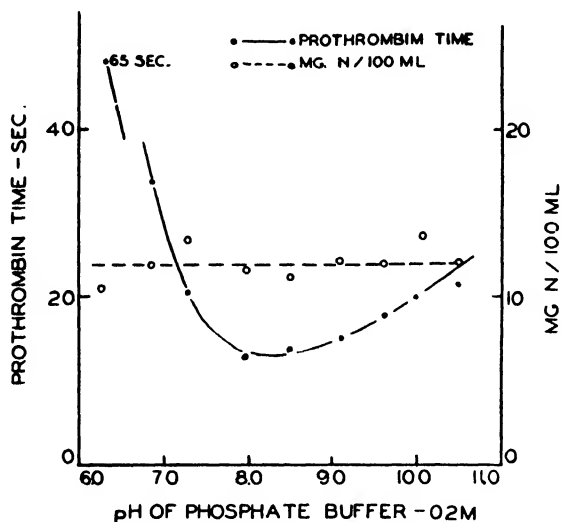


FIG. 3. Effect of the pH of the buffer on the elution of prothrombin from aluminum hydroxide.

8.0 were prepared from mono- and disodium phosphate while those from pH 8.0 to 10.5 were prepared from mono- and trisodium phosphate. It is apparent that a buffer of pH 8.0–8.5 results in the most active prothrombin preparation. All eluates were adjusted to pH 7.4–7.6 as soon as the aluminum hydroxide had been centrifuged down. An attempt was made to use 0.2 M borate buffers over the pH range of 8.0–10.0. Very inactive prothrombin solutions were obtained with the shortest prothrombin time at pH 9.5 instead of at 8.0 as with the phosphate buffers.

*Effect of the Concentration of the Buffer on the Elution of  
Prothrombin from Aluminum Hydroxide*

The prothrombin from 5.0 ml. samples of defibrinated rabbit plasma was adsorbed by 0.3 ml. of aluminum hydroxide. The prothrombin was then eluted with phosphate buffers of pH 8.0 in concentrations of 0.05, 0.10, 0.15, and 0.20 *M*. The data obtained are presented in Fig. 4, from which it is evident that 0.2 *M* phosphate buffer is by far the most

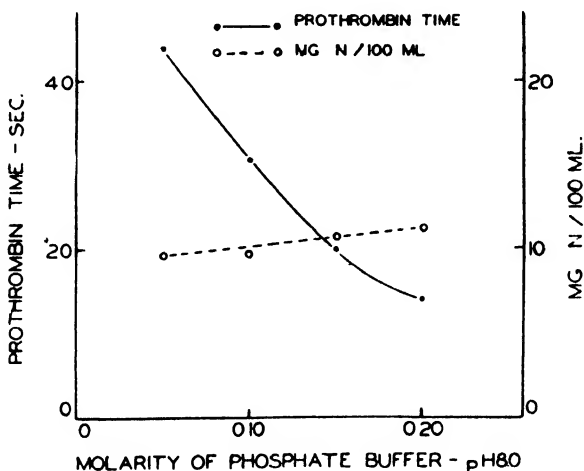


FIG. 4. Effect of the concentration of the buffer on the elution of prothrombin from aluminum hydroxide.

satisfactory buffer concentration with which to elute the prothrombin. We were not able to study the effect of concentrations above 0.2 *M* since this is the upper limit of solubility of sodium phosphate at 0°C.

*Effect of Variation in the Method of Elution and Dialysis on the  
Prothrombin Eluted from Aluminum Hydroxide*

The original conditions for the elution of prothrombin from aluminum hydroxide with phosphate buffer, involving inverting the tube slowly for 10 minutes, followed by dialysis against oxalated saline, had been chosen quite arbitrarily. It was of interest to discover whether a variation in the method of elution, saturation of the prothrombin solution with sulfanilamide before dialysis, and dialysis against dis-

tilled water as suggested by Seegers *et al.* (7) would result in a more active prothrombin solution. The data covering this phase of the investigation are presented in Table II. It is evident that the original conditions of elution, and dialysis against distilled water, produce the most active eluate.

TABLE II  
*Effect of Variations in Method of Elution and Dialysis on  
the Prothrombin Eluted from Aluminum Hydroxide*

Date	Conditions	Dialyzed against	Prothrombin time	N/100 ml. eluate
			<i>sec.</i>	<i>mg.</i>
4-23-46	Inverted for 10 mins., allowed to stand 15 mins.	oxalated		
		saline	14.3	11.6
	Inverted for 10 mins.	oxalated		
		saline	19.5	13.8
9-17-46	Suspended in buffer	oxalated		
		saline	30.2	10.7
	Inverted at 10 min. intervals for 30 mins.	oxalated		
		saline	24.3	9.6
	Inverted at 10 min. intervals for 1 hr.	oxalated		
		saline	22.2	10.5
	Inverted at 10 min. intervals for 2 hrs.	oxalated		
		saline	18.3	9.6
	Inverted for 10 mins., allowed to stand 15 mins.	oxalated		
		saline	17.9	9.7
	Inverted for 10 mins., allowed to stand 15 mins.	distilled water	16.5	7.9
	Inverted for 10 mins., allowed to stand 15 mins., saturated with sulfanilamide	distilled water	17.0	7.6

5.0 ml. plasma mixed with 0.3 ml. aluminum hydroxide. Prothrombin eluted with 5.0 ml. of 0.2 *M* phosphate buffer, pH 8.0.

#### ACKNOWLEDGMENT

We wish to acknowledge the technical assistance of Miss Annabel Avery, B.A.

#### SUMMARY

A method has been presented for the preparation of prothrombin involving its adsorption on aluminum hydroxide followed by elution with 0.2 *M* phosphate buffer, pH 8.0. By this simple procedure it is



possible to prepare a prothrombin solution containing 50% or more of the prothrombin present in the original plasma with a nitrogen content of 4-5 mg./100 ml.

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# Fermentation Studies with Brewers' Yeast.

## I. A Synthetic Medium \*

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Received July 18, 1947

### INTRODUCTION

If it were possible to control, and hence vary at will, all of the constituents of beer wort, experimentation would readily yield valuable information as to the requirements of yeast for fermentation. Obviously beer wort is extremely complex, even though it is usually produced from no more than two or three principal raw materials, *i.e.*, barley malt, some cereal adjunct (usually corn or rice), and hops. To synthesize beer wort is, as yet, impossible. Ultimately, such a synthesis might involve the solution of several hundred substances in water. Although much is known of the composition of beer wort, unknown substances represent by far the majority of its constituents. On the other hand, many of the constituents of beer wort are probably inert and it is conceivable that a synthetic solution embodying most of the active ingredients of wort could be very useful. Such a solution might be used to identify unknown active ingredients of wort and also to study the significance of the known components.

The past decade has seen great progress in the study of bacterial metabolism, most particularly in the field devoted to nutrilites for microorganisms. These studies had their early start with Wildier's bios work and reached fruition with the synthesis of the B vitamins, most of which are now recognized as microbial nutrilites. In view of this progress, it seemed worthwhile to reinvestigate brewery fermentations with the object of determining whether a suitable synthetic medium could now be prepared and utilized in further study. Beside the obvious value of such a study, it was considered quite possible also that the information obtained might ultimately enable the development of a system of assay procedures with yeast as a test organism, and fermentation rates as a measure of response, whereby the concentration, with respect to any of the essential fermentation factors in a given beer wort, could readily be estimated.

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\* Presented before the American Chemical Society at Atlantic City, New Jersey April 15, 1947.

Although brewery fermentations may extend over a period up to 7 days or more, the first 18–24 hours are, in many respects, the most critical. Therefore, first attention was directed to the early phases of fermentation. It is during this period that the yeast converts its environment to one more favorable to itself than to possible infecting organisms; oxygen is consumed with an accompanying shift in redox potential, the yeast reproduces to some extent and enters the rapid phase of fermentation. The brewer knows from experience that a sluggish initial fermentation may be a warning of later difficulties.

The organic factor, or factors, responsible for a stimulation of the rate of fermentation have often been called Z factors, after Euler and Swartz (1). Kögl and Borg (2) have recently studied the influence of Z factors on fermentation by German bakers' yeast. They note that these yeasts differ considerably in response to extracts believed to have Z factor activity. With regard to inorganic factors, Joslyn (3) has reviewed the literature on the mineral metabolism of yeast and finds much that is controversial and conflicting. In the light of the above, it was thought best to begin our studies with a relatively simple medium.

A synthetic medium (No. I, Table I), based largely on preliminary experiments, and in part on the medium described by Atkin, Schultz and Frey (4) in work on bakers' yeast, was first employed.

TABLE I

*Media*

Provisional medium	No. 1		No. 2		No. 3		No. 4	
Ingredient	Per cent w/v	Molarity	Per cent w/v	Molarity	Per cent w/v	Molarity	Per cent w/v	Molarity
NH <sub>4</sub> Cl	0.187 g.	0.035	0.187 g.	0.035	0.187 g.	0.035	0.187 g.	0.035
NaH <sub>2</sub> PO <sub>4</sub> · H <sub>2</sub> O	0.248 g.	0.018	0.248 g.	0.018	0.248 g.	0.018	0.248 g.	0.018
KCl	0.134 g.	0.018	0.134 g.	0.018	0.134 g.	0.018	0.134 g.	0.018
MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.080 g.	0.003	0.080 g.	0.003	0.080 g.	0.003	0.080 g.	0.003
CaCl <sub>2</sub>	—	—	—	—	0.222 g.	0.003	0.222 g.	0.003
Sucrose	5 g.	—	—	—	—	—	—	—
Dextrose	—	—	5 g.	—	5 g.	—	5 g.	—
B <sub>1</sub>	80 γ	—	80 γ	—	80 γ	—	80 γ	—
B <sub>6</sub>	80 γ	—	80 γ	—	80 γ	—	80 γ	—
Niacin	800 γ	—	800 γ	—	800 γ	—	800 γ	—
Casein Hydrolyzate	—	—	—	—	—	—	0.2 g.	—
Citric Acid in Buffer (C <sub>6</sub> H <sub>8</sub> O <sub>7</sub> · H <sub>2</sub> O)	1.68 g.	0.080	—	—	—	—	—	—
Succinic Acid in Buffer (C <sub>4</sub> H <sub>6</sub> O <sub>4</sub> )	—	—	0.59 g.	0.050	0.590 g.	0.050	0.590 g.	0.050

## APPARATUS AND PROCEDURE

*The Fermentometer.* Fermentation tests are conducted in a fermentometer such as that described by Schultz *et al.* (5, 6). This apparatus employs 8 oz. bottles which are rocked continuously at the rate of 180–200 oscillations per minute while submerged in a constant temperature bath at 30°C. The gas evolved as a consequence of fermentation is collected in 500 ml. gasometers which are filled with a 15% solution of sodium sulfate. At each reading, the pressure within the gasometer is adjusted to atmospheric pressure by means of a leveling bulb.

*Reagents. Salt Solutions.* Prepared in volumes such that a suitable aliquot, usually 5 ml., provides the desired concentration in the final medium. Stored at room temperature.

*Vitamin Solutions.* Prepared in convenient concentrations in small volumes and stored at 5°C.

*Sugar Solutions.* Prepared freshly for each run in 50% concentrations.

*Casein Hydrolyzate.* 80 ml. of vitamin-free acid hydrolyzed casein, 10% (General Biochemicals, Inc.), adjusted to pH 5.0 and diluted to 100 ml. Stored at 5°C.

*Buffers.* Sufficient citric acid, or succinic acid, is weighed out to give a concentration of  $M/3$  in one liter of solution. The acid is dissolved in 800 ml. of distilled water and the pH adjusted to 4.9 with 10 *N* sodium hydroxide. It is then made to one liter and stored at 5°C.

*Yeast.* Pressed bakers' yeast in bulk and pressed brewers' yeast from local breweries are received each week and stored at 5°C. The bakers' yeast is stored as received until used, and the brewers' yeast is closely packed in beakers before storage. Immediately before use, 50 g. of the brewers' yeast are suspended in 800 ml. of distilled water with the aid of a Waring Blendor. The Blendor jar is washed with 100 ml. of distilled water. The yeast is then sucked dry on a large Büchner funnel, washed once with 100 ml. of distilled water, and redried. Either 10 or 15 g. of the untreated bakers' yeast or the washed brewers' yeast are creamed with ice cold distilled water and adjusted to a volume of 100 ml. Aliquots of 10 ml. are pipetted into the reaction bottles as the last step before placing the bottles in the fermentometer.

*Procedure.* Aliquots of the various solutions are distributed among the 7 reaction bottles employed in each test, and the volume in each bottle is adjusted to 90 ml. with distilled water. Ten ml. portions of the yeast suspension are added to the series of bottles in order at half-minute intervals. Three minutes are necessary to pipette the yeast and three additional minutes are required to cap the bottles, insert them in the fermentometer and connect the gasometers. The shaker is started immediately and the initial, or "0" reading, is taken two minutes later. Since the complete operation from the pipetting of the first yeast aliquot to the start of the shaker consumes six minutes, the midpoint is arbitrarily accepted as "0" time. Readings of gas volumes are made periodically, usually every half hour. The individual gasometers are read at half-minute intervals to correspond with the intervals between the additions of the yeast aliquots.

*Precision of Measurements.* To estimate the precision of our measurements, 7 replicate fermentations were conducted simultaneously. For

a mean gas volume of 122.4 ml. a standard deviation of 0.976 was obtained. At a mean gas volume of 309.7 ml. the standard deviation was 1.6 ml.

## EXPERIMENTAL

### *Selection of Sugar and Buffer*

Sucrose has often been employed in fermentation investigations. The use of sucrose is undoubtedly related to the fact that it was one of the first sugars obtainable in highly purified form. For our studies, which are designed for eventual application to the analysis of brewery fermentation, there appears to be no good reason for the retention of sucrose as the fermentable sugar. Maltose would ordinarily be the sugar of choice, but pure maltose is fermented by brewers' yeast at a lower rate than dextrose, and furthermore, commercial maltose has been found to be of questionable purity as regards dextrose or dextrin content. Dextrose, which is obtainable in a high state of purity, was adopted for the present investigation.

Atkin *et al.* (4) employed a citrate buffer in their work with bakers' yeast. A succinate buffer is equally effective in the range of pH 4-6.

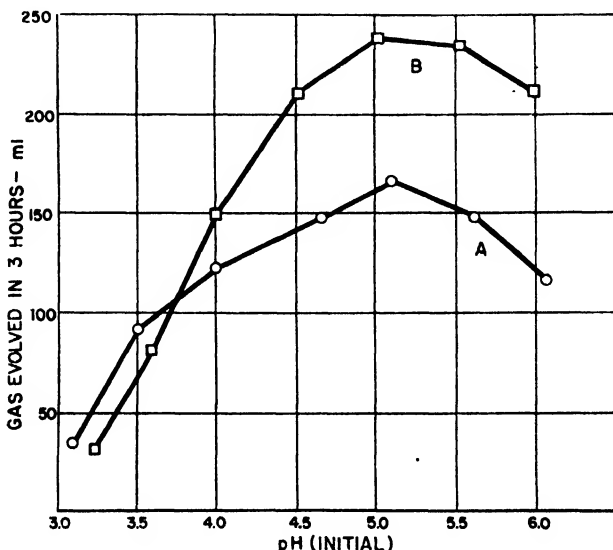


FIG. 1. Influence of pH and buffer on the rate of fermentation of dextrose by brewers' yeast. A. Citrate; B. Succinate.

These two buffers were compared at equimolar concentrations over a range of pH 3-6 with results which are shown in Fig. 1, where the 3-hour gas totals have been plotted against initial pH. It may be observed that the pH optimum appears to lie in the same range (about pH 5.0) with both buffers. The gas evolution with the succinate buffer is significantly higher than in the citrate buffer solutions. To avoid any possible error due to deterioration of the yeast in the interval between these two tests, the tests were made on the same day, using the same yeast, and the citrate series was run first. The apparent adverse effect of citrate, or possible stimulatory effect of succinate, has not yet been investigated in detail, but succinate was accepted as the buffer of choice, and pH 4.9-5.0 as optimum for use in further studies. The pH displacements which occurred in the course of the fermentations were observed to be about the same for both buffers, *i.e.*, approximately 0.05 pH units/100 ml. of gas evolved.

The concentration of buffer employed in Medium No. 1 and in the above tests was somewhat arbitrary, and therefore a series of succinate concentrations at pH 4.9 was tested with results shown in Table II. A

TABLE II  
*Succinate Buffer Concentrations*

Molarity of buffer	0	0.0167	0.0333	0.0500	0.0667	0.0833	0.100
Gas volume, 3 hrs., ml.	317	358	357	355	350	347	341
pH shift	1.9	0.60	0.34	0.22	0.19	0.16	0.12
pH shift/100 ml. gas	0.06	0.16	0.09	0.06	0.05	0.05	0.04

concentration of 0.05 *M* was selected as providing maximum fermentation rates with the least displacement of pH. On the basis of these results, Medium No. 2, containing dextrose and succinate buffer, 0.05 *M* at pH 4.9, was adopted for further experiments.

#### *Sugar Fermented in Terms of Gas Evolved*

Employing Medium No. 2, the sugar equivalents of the gas evolved from dextrose, maltose, and sucrose were estimated. The sugars, in amounts of 0.5 and 1.5 g., were fermented until the cessation of gas evolution. The volumes obtained are shown in Table III, from which

TABLE III  
*Gas Equivalents of Sugars*  
 (Provisional Medium No. 2)

	Sugar	Total gas evolved	Gas evolved/g. sugar
	<i>g.</i>	<i>ml.</i>	
Dextrose	0.5	87	—
	1.5	297	210
Dextrose	0.5	84	—
	1.5	292	208
Sucrose	0.5	92	—
	1.5	312	220
Maltose	0.5	82	—
	1.5	292	210

it may be estimated that the gas equivalent to 1 g. of sugar is roughly 210 ml. for dextrose and maltose and 220 ml. for sucrose. The gas yield from the first 0.5 g. of sugar is lower than that obtained from additional quantities, no doubt because a certain amount of carbon dioxide is required for saturation of the fermentation medium. On the basis of 210 ml. of gas/g. of sugar, it follows that an evolution of 420 ml. of gas corresponds to the fermentation of about 2 g. of sugar, which in turn corresponds to a reduction of 2° Balling. A brewery-type fermentation which is conducted at 10°C. may show a corresponding extent of fermentation in about 24 hours when pitched at an equivalent rate (unpublished data—these laboratories). In most of the experiments herein reported, the gas evolved in three hours amounts to 300–400 ml. and hence the extent of fermentation in these tests at 30°C. roughly approximates that which takes place in a brewery fermentation in the first 24 hours.

#### *Other Constituents of the Provisional Medium*

To estimate the relative importance of the components of the provisional medium, fermentation tests were conducted in which the various factors were singly omitted from Medium No. 2. The results of these tests are shown in Table IV. The most noticeable effects are produced by the absence of  $\text{NH}_4^+$ ,  $\text{PO}_4^-$ ,  $\text{Mg}^{++}$ ,  $\text{SO}_4^-$ , and  $\text{K}^+$ . The omission of thiamin, pyridoxine, and niacin was without significant effect. Sodium and chloride were also without effect at the concentra-

TABLE IV

*Effect of Omission of Various Factors of Provisional Medium No. 2*

Factor omitted	Gas evolved		
	60 minutes	120 minutes	180 minutes
	<i>ml.</i>	<i>ml.</i>	<i>ml.</i>
None (Control)	101	243	416
NH <sub>4</sub>	90	203	319
PO <sub>4</sub>	95	222	370
K	100	240	401
MgSO <sub>4</sub>	97	233	386
B <sub>1</sub>	99	240	410
.....			
None (Control)	100	244	413
* Mg	96	234	397
† SO <sub>4</sub>	98	238	392
Pyridoxine	99	239	409
Niacin	100	242	412
NH <sub>4</sub>	90	197	311

\* To omit Mg above, an equivalent amount of Na<sub>2</sub>SO<sub>4</sub> replaced the MgSO<sub>4</sub>.

† To omit SO<sub>4</sub> above, an equivalent amount of MgCl<sub>2</sub> replaced the MgSO<sub>4</sub>.

tions employed. The difference between these results with brewers' yeast and those reported for bakers' yeast by Atkin *et al.* (4) are rather marked, particularly with regard to the influence of thiamin, magnesium, and phosphate. Considering that other bios factors might be of importance for brewers' yeast, biotin, calcium pantothenate, and inositol (50  $\gamma$ , 1 mg., and 5 mg./100 ml., respectively) were added to the medium, but no noticeable increase in the rate of gas evolution was observed up to three hours, as compared to a control without these factors.

#### *A Comparison of Bakers' and Brewers' Yeasts with Medium No. 2*

To determine whether the formulation of Medium No. 2 had minimized, for brewers' yeast, the thiamin and magnesium effects reported for bakers' yeast by Atkin *et al.* (4), a comparison of the two yeasts was made using this medium, with results which are shown in Fig. 2. It is apparent that bakers' yeast shows a marked dependence on thiamin and magnesium, confirming the results of Atkin *et al.*, whereas brewers' yeast is relatively unaffected.



*Concentration of Ammonia*

Proceeding with a systematic study of the ingredients of the medium, the optimum level of ammonia concentration was determined in a series of tests with results which are shown in Table V. A concentration of 0.035 *M* was indicated as adequate for further use. The choice of

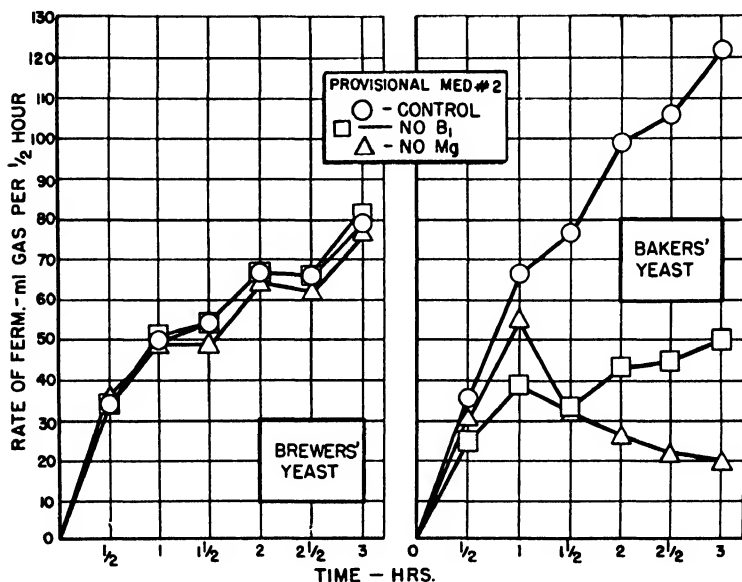


FIG. 2. The influence of thiamine and magnesium on the rate of fermentation of brewers' and bakers' yeasts.

TABLE V  
*Rate of Fermentation as Determined by Gas Evolution  
at Various Concentrations of Ammonia*

Time	ML. gas evolved at ammonia concentrations* of:						
	0	0.00438 <i>M</i>	0.00875 <i>M</i>	0.0175 <i>M</i>	0.035 <i>M</i>	0.070 <i>M</i>	0.140 <i>M</i>
<i>min.</i>							
60	72	78	78	81	80	78	72
120	162	186	186	187	186	179	166
180	255	319	320	322	321	309	283

As  $\text{NH}_4\text{Cl}$ .

0.035 *M* was not based upon the minimal adequate concentration, but rather on the selection of a concentration providing a reasonable excess to avoid deficiencies in cases where a slightly greater concentration should be required.

### *Concentration of Phosphate*

The determination of a suitable phosphate concentration was made in a similar fashion and a concentration of 0.018 *M* was found to be adequate.

### *Concentration of Sulfate*

The concentration of sulfate was similarly determined. Magnesium chloride was substituted for magnesium sulfate in Medium No. 2 in order to maintain the concentration of magnesium unchanged. The concentration of 0.003 *M* was found to be satisfactory.

### *Concentration of Magnesium—Influence of Calcium*

To study the influence of magnesium concentration on the rate of fermentation, potassium sulfate was substituted for magnesium sulfate to maintain the sulfate concentration unchanged, and magnesium chloride was then used as a source of magnesium in Medium No. 2. The results obtained are shown in Table VI, from which it will be seen

TABLE VI  
*Influence of Magnesium \* on the Rate of Fermentation*  
(Provisional Medium No. 2)

Concentration of Mg	Gas evolved		
	60 minutes	120 minutes	180 minutes
<i>M</i>	<i>ml.</i>	<i>ml.</i>	<i>ml.</i>
0	72	171	299
0.003	77	183	321
0.006	78	188	327
0.012	82	192	332
0.024	79	185	325
0.048	75	173	305

\* As  $\text{MgCl}_2$  ( $\text{K}_2\text{S}_4$  substituted for  $\text{MgSO}_4$ ).

that the maximal rate of fermentation is obtained at a concentration of 0.012 *M* with respect to magnesium. This concentration is greater than that employed in Media Nos. 1 and 2 (0.003 *M*). Furthermore, there is a sharp drop in gas evolution as the concentration of magnesium is increased to 0.024 *M* and 0.048 *M*.

Calcium is an important constituent of brewing waters, and the possibility of some interrelationship between calcium and magnesium was considered. Various ratios of calcium and magnesium were tested, with results shown in Table VII. From the data it would appear that

TABLE VII

*Influence of Calcium \* and Magnesium \* on the Rate of Fermentation*  
(Provisional Medium No. 2)

Concentration of Ca	Concentration of Mg	Gas evolved		
		60 minutes	120 minutes	180 minutes
<i>M</i>	<i>M</i>	<i>ml.</i>	<i>ml.</i>	<i>ml.</i>
—	0.003	76	185	310
—	0.012	82	190	316
0.003	0.003	84	198	336
0.012	0.012	80	192	329
0.012	0.003	82	197	335
0.003	0.012	82	192	333

\* Calcium as  $\text{CaCl}_2$ ; magnesium as  $\text{MgCl}_2$ .

$\text{K}_2\text{SO}_4$  used to replace  $\text{MgSO}_4$  in Provisional Medium No. 2.

in this medium a maximal stimulation of fermentation is obtained when both magnesium, 0.003 *M*, and calcium, 0.003 *M*, are present. Calcium ions, in the absence of magnesium, were found to be without effect on the fermentation rate at low concentrations, and somewhat inhibitory at higher concentrations. Another series of magnesium concentrations was tested in the presence of 0.003 *M* calcium in all bottles, with results shown in Table VIII. These data show a maximal effect in the presence of 0.003 *M* calcium from magnesium at 0.003 *M* concentration. The two "titrations" of magnesium (with and without calcium, 0.003 *M*) are compared in Fig. 3, from which it will be seen that calcium appears to eliminate the toxic, or inhibitory, action of magnesium ions at the higher concentrations. In view of these results, the basal medium was further modified to contain calcium chloride, 0.003 *M* (Medium No. 3).

TABLE VIII.

*Influence of Magnesium \* on the Rate of Fermentation  
in the Presence of 0.003 Molar Ca  
(Provisional Medium No. 3)*

Concentration of Mg	Gas evolved		
	60 minutes	120 minutes	180 minutes
<i>M</i>	<i>ml.</i>	<i>ml.</i>	<i>ml.</i>
0	83	193	324
0.003	83	203	356
0.006	86	204	346
0.012	86	207	358
0.024	85	205	358
0.048	82	200	353

\* As  $MgCl_2$ .

$K_2SO_4$  substituted for  $MgSO_4$ .

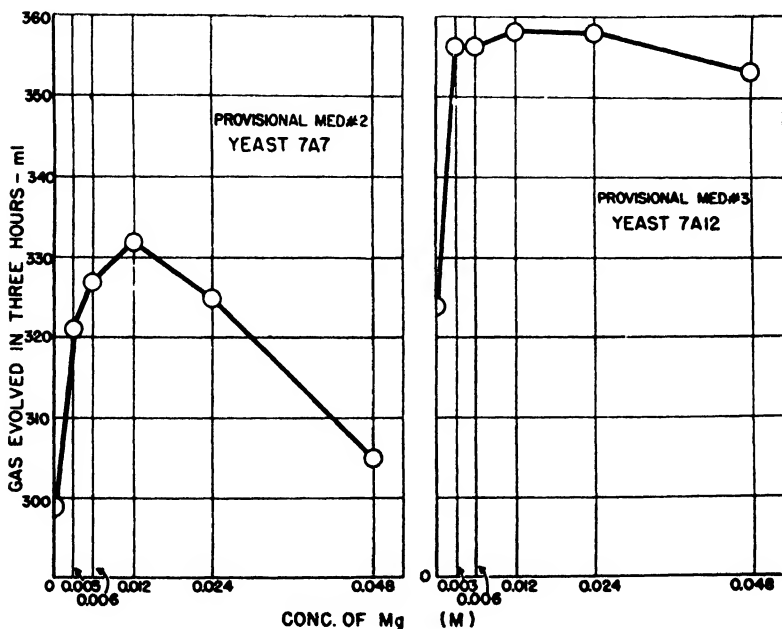


FIG. 3. The effect of increasing concentrations of Mg on the rate of fermentation by brewers' yeast in the absence of Ca (Med. No. 2) and in the presence of 0.003 M  $CaCl_2$  (Med. No. 3).

*Concentration of Potassium*

Using Medium No. 3, the effect of various concentrations of potassium was determined with results which indicate that the concentration of 0.018 *M* is adequate, requiring no change from the concentration previously employed.

*Amino Acid Nitrogen*

Although yeast can utilize ammonium ions as the exclusive source of nitrogen, there is evidence that amino acid nitrogen [Hertz (7)] is more rapidly utilized under certain conditions. Acid-hydrolyzed casein (vitamin-free), of the type used in microbiological vitamin assays, was found to give an increased gas production in our tests. A series of concentrations was tested with results shown in Table IX.

TABLE IX  
*Influence of Hydrolyzed Casein on Rate of Fermentation*  
(Provisional Medium No. 3)

Hydrolyzed casein per cent casein w/v	Gas evolved		
	60 minutes	120 minutes	180 minutes
	<i>ml.</i>	<i>ml.</i>	<i>ml.</i>
0	—	211	366
0.04	81	211	390
0.08	81	211	390
0.12	84	213	389
0.20	84	215	395
1.07	79	205	384

These results and others (not shown) indicated that a quantity of hydrolyzate equivalent to 0.2 g. of casein/100 ml. of medium gave a maximal effect. This quantity of hydrolyzed casein was incorporated into Provisional Medium No. 4.

*Yeast Extract*

A further increase in fermentation rate was observed on the addition of Difco Yeast Extract. The magnitude of the response and the effect of various concentrations are shown in Table X. A slow rise in gas

TABLE X  
*Influence of Yeast Extract on Rate of Fermentation*  
 (Provisional Medium No. 4)

Yeast extract per cent w/v	Gas evolved		
	60 minutes	120 minutes	180 minutes
0	ml. 86	ml. 212	ml. 381
0.05	87	217	395
0.1	92	220	403
0.2	94	225	418
0.4	93	236	422
0.8	93	235	428

production as the quantities are increased beyond 0.2% may be observed.

*Synthetic Medium Compared with Beer Wort*

A comparison of the fermentation rate in beer wort, hopped and unhopped, with various synthetic media, is given in Table XI. The

TABLE XI  
*Rates of Fermentation in Synthetic Medium and Beer Wort*

Basal medium	Sugar per cent w/v	Addition per cent w/v	Gas evolved		
			60 minutes	120 minutes	180 minutes
Beer wort (unhopped)	—	—	ml. 97	ml. 225	ml. 362
Beer wort (hopped)	—	—	90	210	346
Provisional Medium No. 4	Dextrose 5	—	83	222	405
Provisional Medium No. 4	Maltose 5	—	57	168	331
Provisional Medium No. 4	Maltose 4.7	Dextrose 0.3	81	185	348
Provisional Medium No. 4	Dextrose 5	Yeast extract 0.2	92	234	429
Provisional Medium No. 4	Maltose 4.7	Dextrose 0.3 Yeast extract 0.2	85	199	369

beer wort represents a laboratory-made all-malt wort. It may be observed that the synthetic medium No. 4, with dextrose, was fermented at a greater rate than either of the hopped or unhopped beer worts after the first hour. When maltose is the only sugar in the synthetic medium, the rate of fermentation is significantly lower than with beer wort. According to Bishop (8), beer wort may contain 6–19% monosaccharides. The substitution of 6% of the maltose by dextrose did not materially influence the rate of fermentation after the first hour. In the presence of yeast extract, however, the rate of fermentation of the maltose-dextrose mixture approximated that of the malt wort after the second hour.

### *Growth of the Yeast During Fermentation*

In order to characterize the conditions under which the fermentation tests were conducted, the dry weight of yeast and the number of yeast cells were determined before and after a 3-hour period of fermentation in Medium No. 4. The dry weight of the yeast increased by 22% (average of three determinations) over the 3-hour period. Cell counts, disregarding buds, showed no increase at all, but the percentage of cells with buds increased from nil to about 64%. How much of the increase in dry weight is due to individual cell growth and how much is due to the newly formed buds could not be determined, but a significant proportion of the increase in weight is no doubt due to the buds.

### DISCUSSION

It should be stressed that the present study is necessarily preliminary and exploratory in nature. It was not considered desirable to study any single factor exhaustively so long as it was probable that other essential factors were either missing or present in improper proportions.

Schultz, Atkin and Frey (9) found thiamin to be a powerful stimulator of fermentation with bakers' yeast, and as Atkin *et al.* (4) showed, this stimulation is dependent on the simultaneous presence in the medium of magnesium, phosphate, and assimilable nitrogen. According to our results, brewers' yeast does not react to added thiamin, and the effect of the omission of magnesium, phosphate, and assimilable nitrogen, although significant, is not nearly so pronounced as with

bakers' yeast. This difference may be due to an inherent dissimilarity between the two kinds of yeast, or, on the other hand, it may be due to the immediate past history of the two yeasts. Thus, bakers' yeast is grown under conditions which produce a yeast of relatively low thiamin content (15–20  $\gamma$ /g. of yeast solids), whereas brewers' yeast contains on the average 100–125  $\gamma$  of thiamin/g. of solids. A difference in the response to thiamin between bakers' and brewers' yeasts may, therefore, not be surprising. The difference in response to magnesium, phosphate, and nitrogen between the two yeasts may be directly related to the composition of the media in which the yeasts are grown, or may be indirectly related to the thiamin content of the finished yeast. Green, Herbert and Subrahmanyam (10) have shown that the enzyme carboxylase is a protein-magnesium-thiamin pyrophosphate complex of a certain definite composition. A low thiamin synthesis, therefore, might quite conceivably prevent the formation of the complex and thereby reduce the retention of magnesium, phosphate, and nitrogen by the cell.

The importance of most of the mineral constituents of the provisional medium has been known for a long time. The study of fermentation rates in synthetic solutions dates back to Sclator (11). More recently, Thorne (12) has studied the influence of specific sources of nitrogen such as amino acids. The effect of specific concentrations of the various ions has not been studied by the methods or media which we have employed. There is no published evidence for the interrelationship which appears to exist between calcium and magnesium in fermentation which is shown in Tables VII and VIII. These observations require further study. The mineral composition of beer worts may vary rather widely, as shown by the analysis of beer wort ash by Siegfried [Hind (13)]. Analysis of the ash of a beer wort does not necessarily disclose the availability of any particular mineral element because several of the mineral elements may exist in organic combination in the form of complexes or otherwise be unavailable. For this reason, more information is required before the composition of a synthetic medium can be compared accurately with beer wort. In this connection it might be mentioned that the present studies indicate the possibility of providing a rapid means for estimating readily available nutrients of beer wort, both inorganic and organic, by a procedure which is basically similar to familiar microbiological assay methods.



*Synthetic Beer Wort*

It cannot be claimed that any one of the synthetic media which have been described is, in any sense, a replica of beer wort. It was not the immediate objective to devise such a replica, but rather to devise a medium physiologically similar to beer wort, of known composition, which is readily reproducible, and in which the influence of various substances on fermentation may be studied in a more or less uncomplicated fashion. Some success in the attainment of this objective is indicated by the experimental results presented in Table XI, where the comparison was made with beer wort.

Brewery fermentations are conducted at 10°C. or lower, and consequently it is clear that experimentation at 30°C. is purely a matter of expediency. The selection of the higher temperature can be justified only by the increased rapidity with which results are obtained. It is especially necessary in studies of fermentation to keep in mind the great disparity in conditions such as temperature, between the experiment and practice, and it is planned to conduct some of our further studies at lower temperatures, both with the fermentometer and also with the Warburg technique.

*Fermentation vs. Growth*

In the course of this work we have constantly spoken of fermentation as the reaction under study, although it is recognized that a certain amount of yeast growth occurs. The use of fermentation to describe the reaction under our conditions requires some comment. In the past, many fermentation studies have been conducted in simple sugar-phosphate solutions. In such investigations, the presence of assimilable nitrogen was carefully avoided in order to reduce assimilation and consequent growth by the yeast. This technique has served many useful purposes even though assimilation was probably never entirely eliminated, as shown by the work of Winzler and Baumberger (14) and Van Niel and Anderson (15). Practical brewery fermentations, and distillery fermentations, too, always involve considerable assimilation and reproduction. Successful brewery operation demands a moderate growth of yeast in each fermentation cycle to maintain a healthy population of yeast organisms, since a sufficient crop of suitable yeast must be available for reuse. Since the provisional medium which we employed contains virtually all the factors known to be necessary for

growth of yeast, it is not unexpected that some assimilation occurs, even in the short intervals of the test. The conditions selected, however, favor alcoholic fermentation by providing a limited supply of oxygen and elevated concentrations of sugar and yeast. The high rate of fermentation which is obtained precludes the use of "growth" to describe the present reaction. The process, under our conditions, may be described as assimilative fermentation. Obviously, such a process cannot be defined rigorously, as one might define "pure" fermentation or "pure" respiration. On the other hand, assimilative fermentation is a process in daily use in industries whereas "pure" fermentation and "pure" respiration (growth) are little more than hypothetical processes. Because assimilative fermentation is difficult to define rigorously in general terms, emphasis must be placed on the precise experimental conditions.

#### ACKNOWLEDGMENT

The authors wish to acknowledge the able technical assistance of Miss Miriam Feinstein.

#### SUMMARY

The fermentation rate of pressed brewers' yeast was studied with the aid of a fermentometer at 30°C. The relative significance of the components of a provisional synthetic medium was systematically examined and, on the basis of the results, the medium was modified. Dextrose was substituted for sucrose, and a succinate buffer at pH 5.0 replaced the citrate buffer. Calcium was added to the basal medium in a certain ratio to the magnesium concentration. Organic nitrogen, in the form of casein hydrolyzate, was incorporated into the medium. The influence of additional yeast growth (bios) factors and also yeast extract was observed. Finally, the rates of fermentation in a normal beer wort and in a synthetic medium were compared. At several points in the course of this study, the behavior of bakers' yeast was compared with that of brewers' yeast.

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## LETTERS TO THE EDITORS

### The Hypotensive Action of 7-Methylpteroylglutamic Acid

7-Methylpteroylglutamic acid was recently reported (1) to be an effective displacing agent for pteroylglutamic acid in bacterial systems. Subsequently, the same compound was found to be a highly specific inhibitor of dopa decarboxylase (2). Based upon this latter finding, it

TABLE I

*Effect of 7-Methylpteroylglutamic Acid (Intravenous) on Blood Pressure*

Intravenous dose mg./kg.	Effect mm.	Comment
1	No effect	
5	-18 -40	4 min. recovery 30 min. recovery
10	-100  -84 -40 -30 -20 -20	Recovery to within 25 mm. in 6 min.; total in 20 min. Recovery 1 hr. Recovery 1 hr. Recovery 1 hr. Recovery 7 min. Recovery 3 min.
20	-20 -55 -30 -36	Recovery 7 min. Recovery 40 min. Recovery 30 min. Recovery 30 min.
30	-30	Recovery 5 min.
35	-100	Recovery 40 min.
50	-90	Recovery 1 hr. and 35 min.
75	Dead in 4 min.	Respiratory arrest
100	Dead in 4 min.	Respiratory arrest
500 (Orally, cat)	No effect	Observed for 5 hrs.

was assumed that the compound would reduce blood pressure by preventing the decarboxylation of tyrosine, the probable mechanism initiating adrenaline formation.

Table I shows the results obtained in dogs.

The values listed in the above table represent those obtained for initial injection in a single dog.

From the table it can be seen that 7-methylpteroylglutamic acid is a powerful depressor. The compound seems to be ineffective orally and produces a phenomenon like tachyphylaxis in that subsequent doses produce markedly diminished effects.

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*Research Laboratories,  
The National Drug Company,  
Philadelphia, Pa.  
June 27, 1947.*

GUSTAV J. MARTIN  
LEO TOLMAN  
ROBERT BRENDEN

### **The Beneficial Effect of Xanthopterin on Lactation, and of Biotin on Reproduction and Lactation, in Mice Maintained on Highly Purified Diets**

In a previous communication (1), the beneficial effect of pteroylglutamic acid on lactation in mice has been reported. Two other factors suggested themselves for study with respect to their function in reproduction and lactation. One was xanthopterin, because of its relationship to pteroylglutamic acid. The other was biotin, which had been found by Kennedy and Palmer (2) to improve lactation in rats when added to a diet containing 30% of powdered egg albumin. Although the experiments of Vinson and Cerecedo (3) had shown that an addition of biotin to their basal diet containing 25% of purified casein had no effect on lactation, it seemed of interest to ascertain whether the addition of larger amounts of this vitamin would prove beneficial. After completion of the experiments discussed in this paper, Fenton and Cowgill (4) reported a beneficial effect of biotin on lactation in mice.

The diet used was as follows: Purified casein (Smaco), 30; sucrose, 48; Crisco, 10; lard, 5; salts, 5; and Ruffex, 2. To this mixture the following vitamin supplements were

added (per kg.): thiamin, 20 mg.; riboflavin, 20 mg.; pyridoxin, 20 mg.; calcium pantothenate, 40 mg.; choline chloride, 500 mg.;  $\alpha$ -tocopherol, 20 mg.; vitamin A concentrate, 67.5 mg. (67,500 units); and vitamin D (Drisdol), 5,000 units. To this basal diet (R-5a) <sup>1</sup> 200  $\gamma$  of biotin was added to give diet R-36; 20 mg. of pteroylglutamic acid to give diet R-35; 20 mg. of pteroylglutamic acid and 200  $\gamma$  of biotin to give diet R-37; and 5 mg. of xanthopterin to give diet R-38. The mice were put on these diets at weaning.

The results obtained on mice belonging to three different strains are summarized in Table I. Xanthopterin improves lactation from 41% obtained on the basal diet to 82%. The addition of biotin shows its effect in two respects: (1) improvement in reproduction and (2) improvement in lactation. The beneficial effect of biotin may also be seen in that the average litter size and the average weight at weaning were

TABLE I

*The Effect of Xanthopterin and Biotin on Reproduction and Lactation in Mice*

Diet	No. of animals bred	Successful gestation	Average litter size born	Average litter size given to nurse	Average litter size weaned	Average weaning weight	Litters weaned
		<i>Per cent</i>				<i>g.</i>	<i>Per cent</i>
Stock	82	96	6.7	5.5	4.8	9.0	95
R-5(a)	56	57	5.4	5.4	4.2	7.6	41
R-38	24	50	5.8	5.8	4.7	8.0	83
R-35	127	90	6.1	5.0	3.8	8.6	68
R-36	9	89	6.4	5.8	5.2	8.8	75
R-37	36	97	7.2	5.4	4.4	9.5	83

as good on diet R-36, and better on diet R-37, than those obtained with the Rockland stock diet.

This investigation was aided by grants from the John and Mary R. Markle Foundation and the National Vitamin Foundation, Inc.

We are indebted to Dr. E. L. R. Stokstad of the Lederle Laboratories for the pteroylglutamic acid. To him, and to Dr. W. A. Lott of the Squibb Institute for Medical Research our thanks are due for the Xanthopterin. The Borden Company generously supplied the vitamin A concentrate.

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<sup>1</sup> In our previous report (1) the choline chloride supplement, through a typographical error, was omitted.

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*July 2, 1947.*

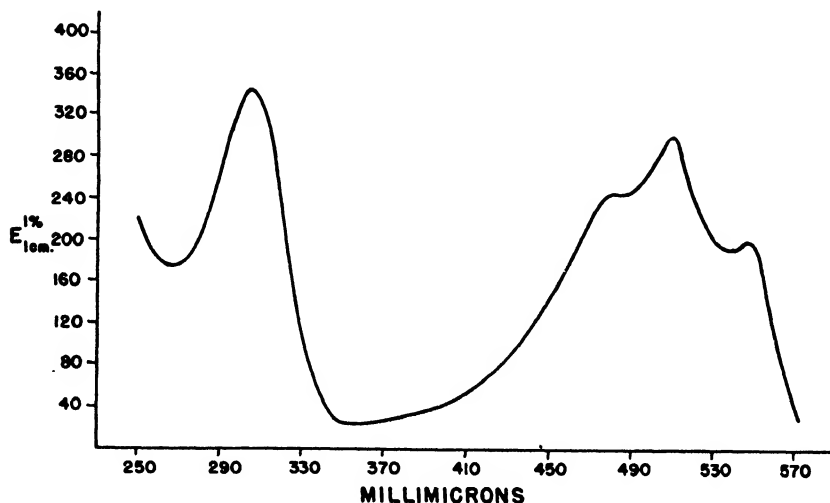
LEONORA MIRONE

LEOPOLD R. CERECEDO

### On the Structure and Possible Functions of a Pigment of *Fusarium Solani* D<sub>2</sub> Purple

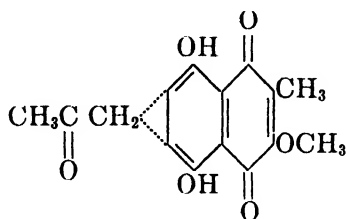
The pigment rubrofusarin, obtained from *Fusarium graminearum* Schwabe, was shown to be capable of influencing the rate of dehydrogenation by *Fusarium lini* Bolley (1). An attempt was now made to identify one of the pigments present in *Fusarium Solani* D<sub>2</sub> purple (2) and to study its possible functions in enzymatic reactions.

The mold was grown on a modified Czapek-Dox medium and the pigment extracted from the dried mycelium after 4 weeks growth with a suitable solvent. It is orange-red, possesses a m.p. of 208°C., and has the empirical formula C<sub>15</sub>H<sub>14</sub>O<sub>6</sub>. The absorption curve of the pigment is given below:



It seems to resemble the absorption curve of Echinochrome A (3). The pigment changes color from an orange, in acid solution, to a

violet, in alkaline solution. It gives a violet color with alcoholic ferric chloride and forms a blue monosodium salt which titrates as a weak acid. It forms a yellow anhydromonoacetate, m.p. 251–252°C. and a colorless triacetate on reductive acetylation. On treatment with reducing agents, the color is discharged from an orange to a light yellow, which, on standing in contact with air, slowly returns. This could indicate an ability to mediate in the course of mechanisms requiring hydrogen transfer. Through certain reactions (4) and degradation studies, it was established that its probable structure may be presented by the following formula:



and it seems, therefore, to be related in structure to that of javanicin (5).

Our interest was directed also toward an understanding of its possible functions, and it was found that even at low concentrations (250  $\gamma$ /l.), with equal amounts of mats formed, the pigment was capable of decreasing the rate of dehydrogenation of isopropanol by *Fusarium lini* B. by about 12%. At higher concentrations (3 mg./l.), an inhibitory action was noticed.

Since both *Fusarium lini* B. as well as *Fusarium lycopersici* are powerful fat-forming molds, experiments were carried out to investigate a possible influence of the pigment on the interrelationship between carbohydrate consumption and fat production. When *Fusarium Solani* D<sub>2</sub> purple is grown on an acetate- instead of carbohydrate-containing Czapek-Dox medium, the pigment formation is almost suppressed, and the fat formation is increased.

When *Fusarium lini* B. is grown on a Raulin-Thom medium containing 2.5, 5 and 10% glucose, to which has been added 1–4 mg. of pigment, it was found that in all cases the growth of *Fusarium lini* B. is suppressed about 50%. However, the presence of the pigment causes an increase in the extent of fat formation at different concentration levels of glucose.



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*Communication No. 58 from the  
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*September 12, 1947*

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## Book Reviews

**Microbiological Antagonisms and Antibiotic Substances.** By SELMAN A. WAKSMAN, Professor of Microbiology in the Rutgers University and Microbiologist in the New Jersey Agriculture Experiment Station. Second edition ix + 415 pp. The Commonwealth Fund, New York, N. Y., 1947. Price \$4.00.

Since the first edition of this book is so well known and was the subject of an extensive review (*Arch. Biochem.* **8**, 156 (1945)), this review will be concerned mainly with the changes made in the second edition. Although considerable progress was made in the subject of antibiotics in the three years elapsing between the first and second editions, the second is only 65 pages larger than the first edition. Such a small increase in size was obtained by omitting material of purely historical importance and that dealing with the clinical applications of penicillins. Additions and deletions were made in each chapter, several tables and plates were omitted and several plates were changed. Among the additions to Chapter 4 were media used in production of penicillin and streptomycin. The summary of chemical and biological properties of some of the more important antibiotic substances was expanded from 3 pages in the first to 5 pages in the second edition. The increase in the number of substances from 22 to 55 reflects, in part, the activity in the antibiotics field in the last four years. One or two of the new substances shows promise of usefulness in medicine. Other chapters have been brought up to date by the addition of pertinent information. Chapter 11, "The Nature of Antibiotic Action," is one of the longest in the book and contains the little that is certain and the much that is speculative about the mode of action of the antibacterial substances. This topic has not received the attention from the physiologists that it deserves. The bibliography of 1053 citations in which the full titles are given is a valuable part of the book.

In certain respects the book is not as complete as one could wish. This, however, is no fault of Dr. Waksman, who can report only what has been published. For example: Table VIII, "Bacteriostatic Spectrum of Penicillin," is identical with Table IX of the first edition and the data were obtained with a penicillin concentrate containing about 2% of the penicillins. It would seem that there has been ample time for such information to be obtained with the several pure crystalline penicillins.

Although obvious errors are few, the range of molecular weights given for the penicillins (p. 207) is about 180 too large. The practice of using *S.* as the abbreviation of the following genera: *Staphylococcus*, *Streptococcus*, *Sarcina*, *Salmonella*, *Serratia* and *Streptomyces* is not recommended even though Dr. Waksman and other microbiologists follow it. The specific epithet alone is not always sufficient to identify a species.

This book is recommended to those who are interested in the antagonisms between microorganisms and in the antibiotic substances.

F. KAVANAGH, New York, N. Y.

**Elsevier's Encyclopaedia of Organic Chemistry. Vol. 14. Tetracyclic and Higher Cyclic Compounds.** Edited by E. JOSEPHY AND F. RADT. Elsevier Publishing Company, Inc., New York and Amsterdam, 1940.  $6\frac{1}{2} \times 10$ , xx + 711 pp. Cloth. Subscription price, \$45; serial price, \$52.50; single volume price, \$60.

This is the first volume to appear of a projected 20 volume encyclopedia the purpose of which is to describe all known organic compounds. It is the first compendium of such broad scope to be published in the English language, and because of the nature of this comprehensive undertaking some comments on the general plan and structure of the encyclopedia as a whole seem necessary. In the descriptive booklet accompanying Vol. 14 the editors point out that each volume of the main work will cover the literature up to four years of the date of appearance. Publication of Vol. 14 was unfortunately delayed because of war conditions. It is estimated that all 20 volumes of the main edition will be available by 1963. Supplements to each volume are promised for each 10 year interval subsequent to its first appearance. It is apparent from the foregoing that the plan of publication is in general similar to that used in the past for Beilstein's Handbuch.

The system of classification adopted is based on the carbon skeleton. All compounds are divided into four series, namely, aliphatic (Vol. 1 through 3), carboisocyclic non-condensed (Vol. 4 through 11), carboisocyclic condensed (Vol. 12 through 14), and heterocyclic (Vol. 15 through 18). A general subject index and group index is to appear in Vol. 19 and the general formula index in Vol. 20. The system of classification employed is similar to, and undoubtedly modeled after, the Beilstein system which is not surprising as the editors had had extensive experience in the Beilstein compilation. The numerous changes in the improved system stem, therefore, from long years of study. Space does not permit an enumeration, much less a discussion, of the general rules adopted, but some major changes in the system may be noted. The arbitrary "system numbers" used in Beilstein have been abandoned. Carboisocyclic compounds (Division III of Beilstein) have been subdivided into condensed and noncondensed series, tending thereby to keep compounds having similar ring systems grouped together. By the elimination of rubrics, and by giving precedence to the number of carbon atoms over saturation, compounds are grouped in somewhat more logical order. Natural products are described throughout the volumes and classified either on the basis of carbon skeleton, if known, or with the first degradation product obtained having a known structure. Such treatment of natural products should make more up-to-date bibliographic material available by bringing them earlier into the system.

The Encyclopaedia of Organic Chemistry has been so planned that biochemical, physiological and pharmacological data are presented along with purely chemical facts, thereby broadening its valuable reference character to investigators in the biological sciences. This objective has been admirably accomplished in Vol. 14 which includes such biologically important groups as the sterols, bile acids, cardiac aglucones, toad poisons, sex hormones, cortical hormones, and cancerogenic hydrocarbons. The sapogenins and the triterpenes also appear in this volume. Along with complete summaries of chemical and biological facts, very complete information is included on methods of degradation and interconversion. This volume, therefore, is also unusually valuable to the specialist working in any of the above fields. It is an excellent compilation both organizationally and factually.

The printing is well done and the binding appears to be of a very durable character, an important item in a laboratory reference work. The size of type employed makes for much easier reading than in other works of this nature. The handling of structural formulae is unusually good. The volume has both subject and formula indices.

If the remaining volumes appear promptly on schedule, the encyclopedia will be invaluable, especially because of its being much more up-to-date than Beilstein. Also the literature survey afforded by such an encyclopedia will be easily complemented by the Decennial Index of Chemical Abstracts (1937-46) which should appear soon. Thus, two works will give complete bibliography, exclusive of current data. The encyclopedia would be *sine qua non* to technical assistants who cannot read German.

J. J. PFIFFNER, Detroit, Mich.

**Données récentes sur la coagulation du sang.** By PIERRE FREDERICQ, Laboratoires de bactériologie de l'université et de la province de Liège. *Actualités Biochimiques*, No. 5. Masson et Cie., Editeurs, Paris; Editions Desoer, Liège, 1946. 64 pp. Price 140 fr.

This short monograph on blood coagulation belongs to a new series, "*Actualités biochimiques*," which in many respects is reminiscent of the well-known brochures published in Paris by Hermann & Cie. before the war.

The discussion is divided into four chapters which deal, in turn, with the first and second phases of blood coagulation, with the pathological clotting disturbances, and with heparin. The selection of literature references appears well balanced. The presentation itself, however, seems somewhat weighted in favor of agents, such as snake venoms, bacterial coagulases, and other enzymes, which, strictly speaking, form no part of the normal clotting mechanism. Thus, no more space is devoted to a discussion of thrombin than to that of staphylococcal coagulase, and the thromboplastic factors present in tissue are treated rather inadequately.

Despite a few reservations, this is an interesting presentation of a difficult subject which may stimulate some to enter this field and others to leave it.

ERWIN CHARGAFF, New York, N. Y.

**Chemistry and Methods of Enzymes**, Second Edition. By JAMES B. SUMNER, Professor of Biochemistry, Cornell University, and G. FRED SOMERS, Plant Physiologist, U. S. Plant, Soil, and Nutrition Laboratories, Ithaca, New York. Academic Press, Inc., New York, N. Y., 1947. xviii + 415 pp. Price \$6.50.

The appearance of the second edition of this book after so short an interval since the first publication indicates the need for works of this type. This second edition is enlarged and in many ways improved. The presentation is clear and the literature references numerous.

The following thoughts, often a matter of personal predilection, occurred to the reviewer: The title, "*Chemistry and Methods of Enzymes*," should perhaps be shortened to "*Chemistry of Enzymes*," since the sections on "methods" consist only of an occasional paragraph or page illustrating how the enzyme chemist goes about his work. This enlivens the reading a great deal, but no one would consult this monograph for technical procedures as might be suggested by the title.

The first part, "General Properties of Enzymes," is a masterpiece of concise presentation which will be most profitable for the novice. Parts II and III list, in 15 chapters, valuable information about a large array of enzymes. The material is so extensive that one, at times, feels as if he is being confronted with a pocket edition of some of the large reference books listed by the authors on p. xi. The material, in most instances, is as well organized as lecture notes, each section being provided with such headings as: Historical, Occurrence, Measurement of Activity, Specificity, *etc.*, which are convenient for reference. However, at times, the placement of topics could be improved: Thus, on p. 26, the conflicting data on peroxidase would seem to fit better in Ch. 9 (Iron Enzymes) than in Ch. 1 (General Properties of Enzymes). Some statements are negativistic. In a few instances the theories of some investigators are discussed, only to be discredited a few lines thereafter, in that they "explain nothing," are "incompatible," have been "contested by many authors," or "shown to be untrue" (pp. 28, 35, 257). It would seem that the errors of the past are too numerous to be discussed in a monograph of this size. Those who have devoted much of their time to the purification of phosphatases will be grieved to find the statement on p. 75 that "nothing especially noteworthy has resulted thus far in most cases."

Particularly excellent, in the descriptive chapters, is the treatment of the enzymes which the senior author has investigated so successfully himself. That enzyme research amounts to more than a mere accumulation of data on one or the other specimen is well illustrated in Ch. 18, on "The Role of Enzymes in Carbohydrate Metabolism and Related Biological Processes." It is refreshing to have this material presented in a way different from the average textbook chapter. However, no one has succeeded, as yet, in presenting carbohydrate metabolism simply enough to make the non-specialist happy. As it is, the average reader will essentially gain respect for the intricacy of the field.

The authors should consider setting aside some space in forthcoming editions for new topics, for example: a coherent discussion of enzymes in relation to the neighboring fields of genetics, immunology, growth, nutrition, bacteriology, and cancerology. A description of some of the modern tools of enzyme research, such as isotopes, spectroscopy, ultrasonic disintegration and other approaches would be very instructive. The senior author is such an authority in the field that he, no doubt, must have significant and guiding ideas about the future of enzyme chemistry, and of these ideas and viewpoints the young generation would like to partake. Maybe in a future edition the "outlook" could be stressed somewhat more than the "retrospect."

The continued success of Sumner and Somers' monograph is certain, partly due to the high quality of the book, partly because there does not exist in the English language any other up-to-date introduction to enzyme chemistry. Anyone who wishes to gain information on enzymes will profit immensely by reading Sumner and Somers' treatise.

F. SCHLENK, Houston, Texas

# $\alpha$ -Amylase from *Bacillus subtilis*. I. Purification and Physical Properties

Frederick J. Di Carlo and Sutton Redfern

*From The Fleischmann Laboratories of Standard Brands, Inc., New York*

Received June 9, 1947

## INTRODUCTION

Despite the extensive industrial application (1) of amylases produced by the *Bacillus subtilis* group, no methods have appeared in the chemical literature for the isolation of these enzymes in a purified condition. Such a procedure is now presented for *B. subtilis*  $\alpha$ -amylase so that the properties of this enzyme may be observed more readily.

## MATERIALS AND METHODS

### *B. subtilis* Amylase Solutions

The enzyme solution available for this investigation was Liquefase.<sup>1</sup> It was prepared by culturing a selected strain of *B. subtilis* on a suitable medium for the time necessary to develop the maximum quantity of amylase. The enzyme is secreted into the medium which is separated from the bacteria. The samples employed contained  $\alpha$ -amylase to the extent of about 2,000 liquefons/ml.

### *Measurement of $\alpha$ -Amylase Activity*

The procedure employed was the Wohlgemuth method as modified by Sandstedt, Kneen and Blish (2) without the use of an excess of  $\beta$ -amylase, since Liquefase contains no detectable quantity of  $\beta$ -amylase. The number 17 varnish color standard described by Redfern (3) was used instead of the dextrin-iodine color standard.

### *Units of $\alpha$ -Amylase Activity*

In agreement with the usual practice of this laboratory, the concentration of bacterial amylase is expressed in terms of the liquefon unit of Józsa and Johnston (4). The dextrinizing procedure was calibrated against the liquefying method of Józsa and Johnston by analyzing a series of samples by both methods. From this calibration

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<sup>1</sup> Trade name, product of Standard Brands, Inc.

the following equation was derived to express the enzyme activity units in terms of liquefons:

$$L = \frac{1430}{w \times t}$$

$L$  = number of liquefons per unit weight or volume,

$w$  = weight or volume of enzyme used for analysis,

$t$  = dextrinizing time in minutes.

#### PURIFICATION OF BACTERIAL $\alpha$ -AMYLASE

Commercial Liquefase was adjusted to pH 6.7–7.0 by the addition of aqueous sodium hydroxide solution. The mixture was clarified by passage through a Sharples supercentrifuge operating at 45,000 r.p.m. for the removal of the bacterial sludge and other insoluble materials. The clear brown solution having a value of 2,070 liq./ml. (1,860 liq./g.) was dialyzed against tap water in a Webcell Continuous Dialyzer<sup>2</sup> so that the output of dialyzed solution approximated 220 ml./hr. The potency of the solution after dialysis was 1,680 liq./ml. These steps involved no appreciable loss of enzyme.

To 18.3 liters of dialyzed solution there was added, with mechanical stirring, two volumes of 95% ethyl alcohol. The stirring was continued for one hour at room temperature and the crude enzyme precipitate was separated by Sharples centrifugation. It was washed in the centrifuge with 65% ethyl alcohol and transferred to a Waring Blendor. The amylase therein was extracted from less soluble materials by 3.2 liters of 1% sodium chloride solution. The extract was kept overnight at 5°C., and centrifuged to yield 3.28 liters of solution which had an enzyme strength of 8,930 liq./ml. The loss of enzyme to this stage was only 5%.

To the centrifuged solution were added 1,975 g. of ammonium sulfate as a precipitant and 33 g. of sodium acetate trihydrate for adjustment of the pH to approximately 6.2. After the mixture was stirred vigorously for two hours, the enzyme was collected in a Sharples supercentrifuge. The enzyme precipitate was washed with 300 ml. of 50% ammonium sulfate solution containing 3 g. of sodium acetate trihydrate.

The wet enzyme precipitate was extracted in a Waring Blendor with 610 ml. of 1% sodium chloride solution. The extract was clarified by vacuum filtration after the addition of some Hyflo Super-cel. The filtrate exhibited a pH of 6.3, and an activity of 17,500 liq./ml.

<sup>2</sup> Brosites Machinery Co., New York, N. Y.

A small amount of toluene was added to the filtrate which was then dialyzed in cellophane tubing using a shaking dialyzer (5) for 18 hours against tap water. The dialyzed solution measured 860 ml., and had a pH of 6.7, and a potency of 11,100 liq./ml. Reprecipitation was carried out by stirring with two volumes of 95% ethyl alcohol for 45 minutes. For facility of removal of the enzyme with minimum mechanical loss, the centrifuge bowl was fitted with parchment paper and the mixture was slowly passed into the operating centrifuge so that the product collected on the paper near the bottom of the bowl whence it was easily removed after withdrawing the paper. The purified amylase, dried *in vacuo* over calcium chloride, was dark brown and weighed 8.0 g. Its potency of 1,003,000 liq./g. corresponded to a 550-fold increase in activity with an over-all yield of 26%.

(In another isolation, material containing 1,300,000 liq./g. was obtained in 48% yield.)

The material prepared as described was further purified by fractional precipitation with acetone. The alcohol precipitate containing 1,003,000 liq./g. was pulverized and 2.5 g. was dissolved in 200 ml. of water. To this solution was added 100 ml. of acetone. The mixture was stirred for 30 minutes and the small precipitate was removed by the centrifugation technique previously mentioned. After washing with 60 ml. of 35% acetone, the solid, dried *in vacuo* over calcium chloride, weighed 0.7 g. and had a potency of 460,000 liq./g.

To the combined centrifuged solution and washing, there was added 100 ml. of acetone. The precipitate was isolated in the same manner as the first fraction and weighed 0.9 g. It was found to have a potency of 1,725,000 liq./g.

A third fraction was obtained after the addition of an equal volume of acetone to the centrifuged solution from the previous step. This fraction weighed 0.2 g. and showed a potency of 394,000 liq./g.

#### PROPERTIES OF PURIFIED ENZYME

*Analyses.* The most potent acetone fraction (1,725,000 liq./g.) was analyzed for various constituents. A sample dried *in vacuo* at 100°C. for 18 hours lost 11.4% of its weight. A residue of 5.3% remained after ashing a portion of this enzyme preparation in a platinum boat. A solution of the ash in hydrochloric acid gave positive tests for ferric and phosphate ions. Micro Kjeldahl analyses showed the enzyme preparation to contain 12.1% nitrogen. When a sample was analyzed for inositol by a modification of the procedure of Woolley (6) it was



found to contain 0.5 mg. of inositol/g. The enzyme preparation displayed no maltase activity as evidenced by its inability to hydrolyze maltose even when employed at high concentrations.

*Action of Ficin.* Merck ficin (200 mg.) was extracted with 10 ml. of water. An amylase solution containing 150 liq./ml. was prepared in a pH 5.0 acetate buffer using enzyme having a potency of 1,003,000 liq./g. To 3 ml. portions of this amylase solution were added: (1) 3 ml. of the ficin solution, and (2) 3 ml. of boiled ficin solution. After storage of both mixtures at 5°C. for 17 hours, the second showed full activity whereas the first had lost 43% of its potency.

*Action of Amylase Inhibitor from Wheat.* The procedure of Militzer, Ikeda and Kneen (7) for the isolation of this inhibitor was modified by substituting vacuum distillation for pervaporation as a means of concentration. The inhibitor so prepared was extremely active against salivary amylase, but had no effect upon our bacterial amylase.

*Enzyme Stability in the Dry State.* It was observed that the residue from the moisture determination displayed a potency of 558,000 liq./g. A number of dry enzyme preparations of widely varying strengths were tested after standing at room temperature for six months. In no instance was a significant change in activity noted.

*pH Effects upon Enzyme Stability.* A stock solution of purified enzyme was prepared in 0.1 M acetate buffer at pH 6.0; its potency was 800 liq./ml. To 5 ml. portions of the stock solution were added 1 ml. volumes of very dilute acetic acid solutions of different concentrations, water, and very dilute sodium hydroxide solutions of various strengths. In this way the desired pH range was covered and the solutions contained only hydrogen, hydroxyl, sodium and acetate as added ions. The solutions were stored in stoppered tubes for 16 hours at 37.5°C. Then pH and activity determinations were made. The results are illustrated in Fig. 1.

*Temperature and pH Effects upon Enzyme Activity.* The effects of pH and temperature on the activity of this purified bacterial amylase were determined by measuring the dextrinizing time at pH values from 3.5 to 10 at temperatures of 30, 40, and 50°C. The buffered substrates were prepared by mixing 20 ml. of 2% soluble starch solution with 5 ml. of modified Britton and Robinson<sup>8</sup> universal buffer. The actual pH values were determined in the digests at the end of the conversions.

<sup>8</sup> The modified buffer was prepared by titrating 100 ml. of a stock solution containing 0.2 M each of monosodium phosphate, acetic acid, and boric acid with 1 N sodium hydroxide to the desired nominal pH value and then diluting to 200 ml.

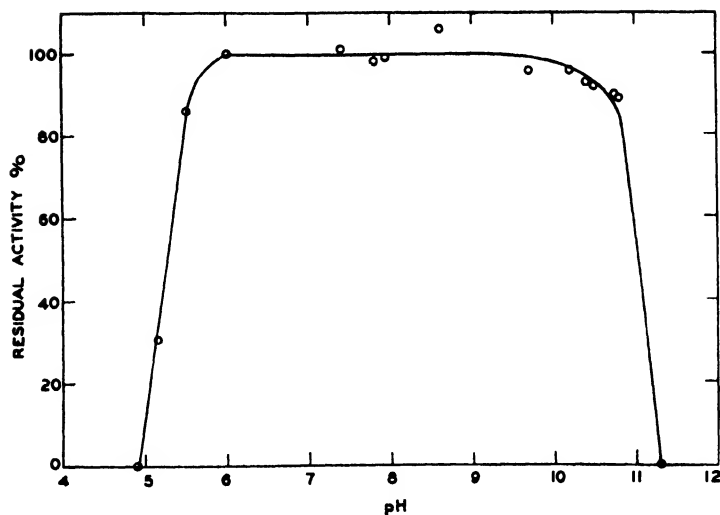


FIG. 1. Stability of bacterial  $\alpha$ -amylase solutions at different pH values.

After attemperating the buffered substrate, 5 ml. of an appropriately diluted enzyme solution was added, and the dextrinizing time determined in the usual manner. The quantity of enzyme used was chosen so

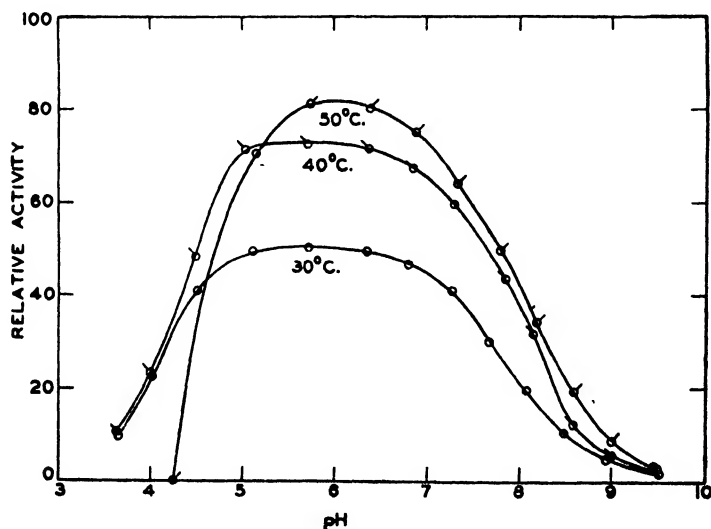


FIG. 2. Activity of bacterial  $\alpha$ -amylase solutions at different temperatures and pH values.

that the dextrinizing times varied between 10 and 30 minutes, thus minimizing the time factor at the higher temperatures where the enzyme would be destroyed.

The data for bacterial amylase are given in Fig. 2.

*Behavior upon Dialysis.* Ten grams of dry enzyme (65,800 liq./g.) was dissolved in 100 ml. of water and dialyzed in cellophane tubes in the shaking dialyzer for 20 hours against tap water. A similar experiment was conducted in which deionized water<sup>4</sup> was employed. The solution dialyzed with tap water lost 13% of its activity whereas the use of deionized water resulted in a 47% decrease. The loss of activity after dialysis against tap water in this experiment was atypical. It has been observed in other experiments carried out under these conditions that the losses approximated 5% regardless of the purity of the enzyme preparation.

Dry bacterial amylase of potency 1,300,000 liq./g. was dissolved in 0.1 *M* acetate buffer at pH 6.0 to produce a solution which analyzed 750 liq./ml. A few drops of toluene was added to 50 ml. of this solution and dialysis was carried out in cellophane tubing against 4 volumes of distilled water at 5°C. The 4 volumes of water was changed at intervals of 24 hours for 4 days, and then after 72 hours. The various dialyzates were separately concentrated to small volumes under reduced pressure below 35°C. Samples of the dialyzed enzyme solution were also removed at these intervals and analyzed for residual enzyme activity. The data are recorded in Table I.

TABLE I  
*Dialysis of  $\alpha$ -Amylase Solution against Distilled Water*

Dialysis hrs.	Potency* liq./ml.	Loss per cent
0	750	0
24	700	7
48	545	27
72	260	65
96	247	67
168	221	70

\* Corrected for changes in volume during analysis.

To test for a coenzyme each sample of dialyzed enzyme solution was analyzed: (1) after the addition of an equal volume of the original enzyme solution which had been inactivated by boiling, and (2) after

<sup>4</sup> The water was deionized by an Illco-Way Deionizer, a product of the Illinois Water Treatment Co., Rockford, Ill.

the addition of a volume of the appropriate concentrated dialyzate sufficient to reconstitute the composition of the original enzyme solution. It was found that neither boiled enzyme solution nor concentrated dialyzate reactivated any of the dialyzed enzyme solutions.

Since Biolase, a bacterial amylase, has been reported to be adsorbed upon cellophane (8) the possibility of explaining the preceding results on this basis was checked. Some of the enzyme solution employed in the previous experiment was allowed to stand in contact with strips of cellophane for 8 days at 5°C. None of the enzyme activity was lost from the solution by this treatment. Another portion of the enzyme solution was dialyzed with mechanical agitation for 3 days against running tap water (97% recovery) and then stored at 5°C. for 9 days with cellophane strips. Again the solution lost no activity.

A sample of Liquefase was adjusted to pH 6.8 and clarified by supercentrifugation. The brilliant amylase solution was transferred to a cellophane tube and dialyzed in the shaking dialyzer for 72 hours against running tap water. One ml. portions of the dialyzed solution were diluted to 100 ml. volumes with 0.025 *M* solutions of various salts and with tap, distilled, and deionized water. After 1 hour at room temperature, the diluted enzyme solutions were analyzed by the dextrinizing procedure. This experiment was run three times. The data recorded in Table II are averages. Since the highest activity was

TABLE II  
*Effect of Salts upon Dialyzed Liquefase*

Diluent	Full activity per cent
0.025 <i>M</i> CaCl <sub>2</sub>	100
0.025 NaCl	95
0.025 Acetate buffer (pH 6.0)	87
0.025 Phosphate buffer (pH 6.8)	94
Water, tap	85
Water, distilled	60
Water, deionized	<10

observed after dilution with 0.025 *M* calcium chloride solution (the diluent employed routinely), this was considered to represent full activity and the values obtained from other diluents were calculated on this basis. The loss of activity upon dialysis was 8%. It must be mentioned that undiluted dialyzed solutions were stable for periods of more than 3 weeks at 5°C.

To ascertain whether the salts exerted stabilizing or activating influence upon dialyzed enzyme solutions, the following experiment was carried out. An amylase solution was dialyzed for 3 days as previously described. Four 1 ml. samples of the dialyzed solution were transferred to 100 ml. volumetric flasks. One was diluted to volume with 0.025 *M* aqueous calcium chloride. The others were diluted with deionized water, and one of these diluted enzyme solutions was analyzed immediately with a substrate containing 5 ml. of 0.025 *M* calcium chloride solution in addition to the usual 20 ml. of buffered starch solution. The other diluted amylase solutions were analyzed after standing 30 minutes at room temperature; one of those diluted with deionized water was tested with starch containing calcium chloride as above. The results are given in Table III.

TABLE III  
*Attempted Reactivation of Diluted Dialyzed Amylase Solution*

Diluent	Standing time	Substrate	Activity
0.025 <i>M</i> CaCl <sub>2</sub>	30 min.	Usual	liq./ml. 1090
Deionized water	30 min.	Usual	520
Deionized water	30 min.	Usual + CaCl <sub>2</sub>	495
Deionized water	<1 min.	Usual + CaCl <sub>2</sub>	1110

### DISCUSSION

The procedure for purification resulted in a 900-fold increase in  $\alpha$ -amylase potency. Although we know that the highest strength attained does not represent the potency of the pure enzyme, the product is considered quite satisfactory for investigation. Further purification studies are in progress. This enzyme protein is quite resistant to denaturation by agitation and by contact with high concentrations of alcohol, even for prolonged periods of time. These properties favored easily obtainable good yields.

The nitrogen content of approximately 12% is somewhat low for a pure protein, but does indicate a fairly high removal of non-protein materials. The hydrolysis by ficin further defines the amylase as protein in nature.

A purified pancreatic  $\alpha$ -amylase prepared by Caldwell was analyzed by Williams *et al.* (9) and found to contain 4.1–4.4 mg. of inositol/g. of enzyme. Since our purified bacterial amylase was found to contain

0.5 mg. of inositol/g. we prefer to consider it an impurity rather than a constituent of the enzyme molecule.

The lack of inhibition of this bacterial amylase by the wheat inhibitor of Kneen and Sandstedt (10) is in agreement with the findings of those workers. The enzyme may be classified with the non-saccharifying type of *B. subtilis* amylase, or so-called commercial type of bacterial amylase (11).

The dry purified *B. subtilis* amylase preparations displayed a remarkable degree of thermal stability. Only 68% of the activity was lost after 17 hours at 100°C. *in vacuo*. Purified *B. macerans* amylase has been reported by Tilden, Adams and Hudson (12) to lose approximately 35% of its activity after 79 days at 5°C.

The stability of *B. subtilis*  $\alpha$ -amylase solutions over the wide pH range of 5.8–10.8 under the conditions described is rather unusual.

This enzyme shows optimum activity over the pH range of 5–6.5 at 30°C. and 40°C.; the optimum becomes much sharper and shifts to pH 6.0 at 50°C. This shifting is due to the extreme sensitivity of the amylase to pH values less than 4.5. This may also be observed in Fig. 1 which depicts the effect of pH upon enzyme stability.

The optimum activity pH range reported herein differs somewhat from the value of 6.5–8.0 given by Wallerstein (1) for a presumably similar bacterial amylase. There is better agreement with the incomplete data of Hollenbeck and Blish (13). The optimum pH range is higher than that of 4.4–4.6 reported for barley malt  $\alpha$ -amylase (14, 15).

Dialysis experiments similar to those of Neubeck and Smythe (16) provided no evidence for the existence of a coenzyme. The progressive decline of enzyme activity upon dialysis may be attributed to denaturation in the absence of a sufficiently concentrated ionic environment.

It has been observed that dialyzed solutions of *B. subtilis* amylase were rendered less stable by dilution with water. Thirty minutes after dilution to 100 volumes with deionized water, the enzyme solution had lost more than half of its dextrinizing power, and at the end of an hour there remained less than a tenth of the original activity. Similar dilutions with tap and distilled water consistently resulted in less extensive losses. The use of certain dilute salt solutions as diluents further limited decreases in activity. Amylase solutions which displayed less activity after dilution with deionized water were not reactivated by the addition of calcium chloride. The effect of this reagent appears to have been stabilization.

## ACKNOWLEDGMENTS

The help of Mr. Herbert Kothe and Mr. Gerald Fox in carrying out the analytical work is gratefully acknowledged.

## SUMMARY

1. A method is described for the isolation of *B. subtilis*  $\alpha$ -amylase of high potency. By this method a 900-fold increase in enzyme activity was achieved.

2. The pH stability and the pH activity curves of the enzyme were determined.

3. The existence of a coenzyme of the bacterial  $\alpha$ -amylase could not be established by experiments in dialysis.

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## $\alpha$ -Amylase from *Bacillus subtilis*.

### II. Essential Groups

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Received June 9, 1947

#### INTRODUCTION

Weill and Caldwell (1) have demonstrated that free sulfhydryl and tyrosine groups are essential for  $\beta$ -amylase activity. Primary amino groups have been found necessary for the functioning of pancreatic  $\alpha$ -amylase, but sulfhydryl groups have been shown to be non-essential (2).

Since bacterial  $\alpha$ -amylases have not been characterized with respect to their essential groups, it was considered of interest to study the effects of a variety of enzyme inhibitors upon purified  $\alpha$ -amylase prepared from *B. subtilis* (3).

#### MATERIALS AND METHODS

The enzyme solutions employed in this investigation were prepared from samples of purified material which ranged in potency from 1,003,000 to 1,910,000 liq./g. .

It was observed that solutions of the bacterial amylase in 0.1 *M* acetic acid-sodium acetate buffer at pH 6.0 were stable to heating at 37.5°C. for periods in excess of 16 hours. Such enzyme solutions were treated with the specified reagents under the conditions reported below, and the inhibiting effects of the reagents upon the dextrinogenic activity of the enzyme were determined as previously described (3). Solutions of reagents capable of changing the pH of the buffered enzyme solutions were adjusted prior to their addition. In some experiments it was desirable to work at a pH lower than 6.0 for inhibition. To prevent inactivation by the acidic medium after completion of treatment by the particular reagent, the pH was adjusted to 6-7 before analysis.

#### *Action of Metallic Ions*

The ability of silver ions to inactivate solutions of *B. subtilis*  $\alpha$ -amylase over a period of one week at room temperature was determined. Silver nitrate was added to solutions at two levels of enzyme concentration to produce  $10^{-3}$  *M*  $\text{Ag}^+$  solutions. The enzyme solutions were buffered at pH 6.0 with 0.1 *M* acetate. The slow inactivation effected by silver ions is shown in Table I. The controls lost about 5% of their activity after 7 days.



TABLE I

*Effect of  $10^{-2}$  M  $\text{Ag}^+$  on Bacterial Amylase at Room Temperature and pH 6.0*

Time hrs.	Residual activity	
	Solution A Per cent	Solution B Per cent
0	100 <sup>a</sup>	100 <sup>a</sup>
2	73.3	90.0
5	50.0	81.2
24	52.2	62.5
48	42.2	35.0
168	6.7	12.5

<sup>a</sup> The original activities of solutions A and B were 450 liq./ml. and 800 liq./ml. respectively.

TABLE II

*Effect of Metallic Ions on Bacterial Amylase in 16 Hours at 37.5°C. and pH 6.0*

Ion	Molarity	Original potency of solution	Residual activity
		liq./ml.	per cent
$\text{Ag}^+$	$10^{-2}$	900	19
$\text{Ag}^+$	$10^{-3}$	900	52
$\text{Ag}^+$	$10^{-4}$	900	89
$\text{Ag}^+$	$10^{-6}$	900	92
$\text{Hg}^{++}$	$10^{-2}$	900	0
$\text{Hg}^{++}$	$10^{-3}$	900	44
$\text{Hg}^{++}$	$10^{-4}$	900	84
$\text{Hg}^{++}$	$10^{-6}$	900	100
$\text{Pb}^{++}$	$10^{-2}$	900	36
$\text{Pb}^{++}$	$10^{-3}$	900	79
$\text{Pb}^{++}$	$10^{-4}$	900	90
$\text{Pb}^{++}$	$10^{-6}$	900	90
$\text{Cu}^{++}$	$10^{-2}$	800	17
$\text{Cu}^{++}$	$10^{-4}$	800	95
$\text{Zn}^{++}$	$10^{-2}$	600	18
$\text{Zn}^{++}$	$10^{-3}$	600	65
$\text{Fe}^{+++}$	$10^{-2}$	600	47
$\text{Fe}^{+++}$	$10^{-3}$	600	100

Table II lists the inactivations produced by several metallic ions at various concentrations. Ions such as  $\text{Na}^+$ ,  $\text{NH}_4^+$ , or  $\text{Ca}^{++}$  were without effect.

*Action of Oxidizing Agents*

The results obtained by treatment of amylase solutions with a variety of oxidizing agents are recorded in Table III.

TABLE III

*Effect of Oxidizing Agents on Bacterial Amylase  
in 16 Hours at 37.5°C. and pH 6.0*

Reagent	Molarity	Original potency of solution	Residual activity
		<i>liq./ml.</i>	<i>per cent</i>
KMnO <sub>4</sub>	10 <sup>-2</sup>	800	< 25
K <sub>3</sub> Fe(CN) <sub>6</sub>	10 <sup>-1</sup>	800	66
K <sub>3</sub> Fe(CN) <sub>6</sub>	10 <sup>-2</sup>	500	90
K <sub>3</sub> Fe(CN) <sub>6</sub>	10 <sup>-3</sup>	500	100
I <sub>2</sub>	10 <sup>-2</sup>	450	0
I <sub>2</sub>	10 <sup>-3</sup>	450	52
I <sub>2</sub>	10 <sup>-4</sup>	450	92
Quinone	10 <sup>-1</sup>	700	0
Cystine	10 <sup>-2</sup>	600	100

It was observed that cupric ions catalyzed oxidation of the enzyme by air (*cf.* Table IV). Bubbling air through enzyme solutions at room temperature for one hour produced no inhibiting effect, but when this operation was carried out in the presence

TABLE IV

*Copper Ion Catalysis of Oxidation of Bacterial Amylase by Air*

Reagent	Cu <sup>++</sup>	Original potency	Residual activity
	<i>M</i>	<i>liq./ml.</i>	<i>per cent</i>
Air	—	400	100
Cu <sup>++</sup>	10 <sup>-2</sup>	400	75
Cu <sup>++</sup> + Air	10 <sup>-2</sup>	400	58

of 10<sup>-2</sup> *M* Cu<sup>++</sup> the loss in activity was marked. A solution of enzyme allowed to stand un-aerated at room temperature for one hour with 10<sup>-2</sup> *M* Cu<sup>++</sup> suffered less extensive inactivation.

*Action of Maleic Acid*

After storage of an amylase solution (800 liq./ml.) for 16 hours at 37.5°C. with 10<sup>-2</sup> *M* maleic acid at pH 6.0, inhibition occurred to the extent of 19%.

### *Action of Iodoacetic Acid*

The treatment of enzyme solutions (500 liq./ml.) with iodoacetic acid under the conditions stated above for maleic acid yielded the following results:  $10^{-1}$  *M* iodoacetic acid, 80% inactivation;  $10^{-2}$  *M* iodoacetic acid, no inactivation.

### *Action of p-Chloromercuribenzoic Acid*

At pH 6.0, the bacterial amylase was not inactivated in a solution saturated with *p*-chloromercuribenzoic acid after 32 hours at 37.5°C. At pH 4.7 and 5°C., enzyme solutions (800 liq./ml.) saturated with this reagent lost activity as follows: 11% after 1 hour, 31% after 3 hours, and 58% after 6 hours. With the same treatment, a more dilute enzyme solution (100 liq./ml.) lost activity much faster: 0% after 15 minutes, 58% after 45 minutes, and 100% after 2 hours. The controls in these experiments withstood the low pH without loss of potency.

### *Action of Carbonyl Reagents*

Hydrazine, phenylhydrazine, and semicarbazide inhibited amylase action as recorded in Table V.

TABLE V  
*Effect of Carbonyl Reagents on Bacterial Amylase  
in 16 Hours at 37.5°C. and pH 6.0*

Reagent	Molarity	Original potency	Residual activity
		liq./ml.	per cent
Hydrazine	$10^{-2}$	700	83
Phenylhydrazine	$10^{-2}$	700	74
Semicarbazide	$10^{-2}$	700	13

### *Action of Nitrous Acid*

The inhibiting effects of nitrous acid under different experimental conditions are summarized in Table VI.

### *Action of Diverse Anions*

Oxalate and fluoride ions were observed to be inhibitory. Under the same conditions employed for maleic acid,  $10^{-2}$  *M* oxalic acid reduced the dextrinogenic activity by 69%, and  $10^{-2}$  *M* fluoride produced 9% inactivation. Similar experiments showed the following ions in  $10^{-2}$  *M* solutions to be inactive: azide, citrate, tartrate, phthalate, salicylate, thiosulfate, sulfide, and cyanide.

### *Reactivation Experiments*

To accomplish rapid inactivations, rather dilute solutions of the amylase were used. Enzyme solutions which showed activity below 200 liq./ml. were found subject to

TABLE VI  
Effects of Nitrous Acid on Bacterial Amylase

HNO <sub>2</sub>	pH	Temp.	Time	Original potency	Residual activity
<i>M</i>		$^{\circ}\text{C.}$	<i>hrs.</i>	<i>liq./ml.</i>	<i>per cent</i>
1	6.0	37.5	16	800	30
10 <sup>-1</sup>	6.0	37.5	16	800	84
10 <sup>-1</sup>	6.0	5	16	730	100
10 <sup>-1</sup>	4.6	5	16	730	9 <sup>a</sup>
1	4.9	5	½	70	80
1	4.9	5	1	70	65
10 <sup>-1</sup>	4.9	5	½	70	100
10 <sup>-1</sup>	4.9	5	1	70	89
10 <sup>-2</sup>	4.9	5	½	70	100
10 <sup>-2</sup>	4.9	5	1	70	95

<sup>a</sup> The control in this experiment lost 43% of its activity.

marked inactivation by 10<sup>-2</sup> *M* Ag<sup>+</sup> and by 10<sup>-2</sup> *M* iodine in 15 minutes at room temperature. (These enzyme solutions were buffered at pH 6.0 with 0.1 *M* acetate.) For reactivation trials, hydrogen cyanide, cysteine, or hydrogen sulfide was added in such quantity that the molar ratio of "reactivating agent" to inhibitor was 10 to 1, and the mixtures were analyzed after an additional 30 minutes at room temperature.

The saturation of dilute enzyme solutions (about 100 liq./ml.) with *p*-chloromercuribenzoic acid was followed by extensive inactivation after 45 minutes at room temperature. As previously indicated, the pH employed in conjunction with this reagent was 4.7. Reactivations were attempted with the same concentrations of the

TABLE VII  
Reactivations of Bacterial Amylase Solutions Inhibited  
by Certain Sulfhydryl Reagents

Inhibitor	Residual activity	Reactivating agent	Residual activity
	<i>per cent</i>		<i>per cent</i>
Ag <sup>+</sup>	48	HCN	100
Ag <sup>+</sup>	48	Cysteine	100
Ag <sup>+</sup>	48	H <sub>2</sub> S	<27
I <sub>2</sub>	67	HCN	100
I <sub>2</sub>	67	Cysteine	69
I <sub>2</sub>	67	H <sub>2</sub> S	71
<i>p</i> -ClHgC <sub>6</sub> H <sub>4</sub> COOH	51	HCN	100
<i>p</i> -ClHgC <sub>6</sub> H <sub>4</sub> COOH	51	Cysteine	100
<i>p</i> -ClHgC <sub>6</sub> H <sub>4</sub> COOH	51	H <sub>2</sub> S	48

reagents used after  $\text{Ag}^+$  and iodine treatments. During the reactivation period of 30 minutes, the pH was raised to 5.5 to prevent enzyme denaturation.

The data obtained are recorded in Table VII.

The inactivations of bacterial amylase in solutions containing 40 liq./ml. (0.1 *M* acetate buffered at pH 4.8–4.9) produced by 1 *M* nitrite at 5°C. were considered satisfactory for reactivation trials. These conditions are comparable to those employed in connection with  $\beta$ -amylase (1). After 20, 40, and 60 minutes, samples were removed, and without delay hydrogen sulfide was bubbled vigorously for 5 minutes through the enzyme solutions. During this treatment the pH was maintained at 6.8 by the addition of 1 *M* sodium acetate solution. Activities were determined after a period of 45 minutes at room temperature. The results appear in Table VIII.

TABLE VIII

*Attempted Reactivations of Bacterial Amylase Solutions  
Inhibited by Nitrous Acid*

Inhibitor	Per cent residual activity after			Reactivating agent	Residual activity per cent		
	(a) 20 min.	(b) 40 min.	(c) 60 min.		(a)	(b)	(c)
—	100	98	93	$\text{H}_2\text{S}$	95	90	85
$\text{HNO}_2$ , 1 <i>M</i>	80	69	59	$\text{H}_2\text{S}$	72	69	39

## DISCUSSION

The inactivations by ions of heavy metals, oxidizing agents, and the more specific reagents *p*-chloromercuribenzoic, iodoacetic, and maleic<sup>1</sup> acids coupled with the reactivations after  $\text{Ag}^+$  inactivation (by hydrogen cyanide and by cysteine), after iodine inactivation (by hydrogen cyanide), and after *p*-chloromercuribenzoic acid inactivation (by hydrogen cyanide and by cysteine) are interpreted as constituting conclusive evidence for the existence of essential free sulfhydryl groups in *B. subtilis*  $\alpha$ -amylase. This conclusion serves to accentuate the difference between bacterial and pancreatic  $\alpha$ -amylases since the latter enzyme is not dependent upon intact free sulfhydryl groups (2).

The mechanism of inhibition by carbonyl reagents has not been elucidated. Other enzymes reported to be inactivated by these reagents include diamine oxidase (6), histidine carboxylase (7), and glutamic acid decarboxylase (8). It has been suggested by Singer (9) that a

<sup>1</sup> This may be attributed to the formation of an addition compound between maleic acid and free sulfhydryl groups in the protein molecule (4, 5).

prosthetic group or an unknown amino acid may be responsible for this effect. It is also conceivable that enzymes inhibited by carbonyl reagents may be mucoids or glycoproteins. The inhibitory effects of the carbonyl reagents are the subject of further investigation.

Nitrous acid is capable of interaction with sulfhydryl, amino, and tyrosine groups. The oxidizing action of this reagent upon sulfhydryl groups is reversible (1); the reactions of nitrous acid with amino and tyrosine groups are irreversible. The failure of hydrogen sulfide to reactivate bacterial amylase, inhibited even to a small extent by nitrous acid, suggests that sulfhydryl groups are not involved. Iodine is considered a specific reagent for essential tyrosine groups in proteins which do not contain sulfhydryl groups necessary for activity. The complete reactivation by hydrogen cyanide of iodine-inactivated bacterial amylase precludes the possibility that a tyrosine group is involved. Therefore, the inactivation of *B. subtilis*  $\alpha$ -amylase caused by nitrous acid may be interpreted as indicative of essential free amino groups rather than as evidence of the presence of essential sulfhydryl or tyrosine groups.

Fluoride and oxalate ions have been reported to inhibit some calcium-requiring enzymes by compound formation with the prosthetic groups (10). These reagents were also observed to inactivate the bacterial amylase, although no evidence of a prosthetic group has been found (3). Solutions of *B. subtilis* amylase have been found to lose activity upon dialysis against either distilled or deionized water, whereas identical solutions displayed stability to tap water dialysis (3). These findings may be another manifestation of the long recognized stabilization of  $\alpha$ -amylases by calcium ions (11, 12), and the decline of activity which occurred during dialyses against purified water may be ascribed to the removal of these ions from the enzyme solutions, followed, perhaps, by denaturation.

The inability of sulfide, cyanide, or azide to inactivate the amylase provides evidence for the absence of copper or iron as constituents of the enzyme system (13, 14, 15). Therefore, the iron found in the enzyme ash (3) must be considered as a contaminant.

#### ACKNOWLEDGMENTS

The help of Mr. Herbert Kothe and Mr. Gerald Fox in carrying out the analytical work is gratefully acknowledged.

## SUMMARY

1. The presence of essential sulfhydryl groups in *B. subtilis*  $\alpha$ -amylase was established.
2. Evidence for the presence of essential amino groups is presented.
3. Inactivations by certain organic carbonyl reagents are reported.
4. Inactivations by fluoride and oxalate ions are considered attributable to removal of calcium ions.

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# The Estimation of Small Amounts of Fatty Acid in the Presence of Polyoxyethylene Sorbitan Partial Fatty Acid Esters ("Tween") and of Serum Proteins

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Received June 9, 1947

## INTRODUCTION

It was recently reported from this laboratory (1, 2) that submerged growth of tubercle bacilli in liquid media is facilitated by a standardized commercial polyoxyethylene derivative of sorbitan monooleate, marketed under the trade mark "Tween" 80,<sup>2</sup> and that serum albumin permits initiation of growth by small inocula. In addition to these beneficial effects, however, it was observed that "Tween" 80 is somewhat inhibitory to tubercle bacilli, and becomes more toxic on standing in aqueous solution, especially in the presence of the commercial bovine serum albumin (Armour) used in the medium. These observations could be explained by hydrolysis of the "Tween" 80, accelerated by lipase in the serum albumin. To verify this interpretation (3, 4) it was necessary to develop an analytical method for estimating small amounts of fatty acid in the presence of large amounts of "Tween" and of serum albumin. Because of the interest of the "Tween" for several fields in biology, as well as the possible general applicability of this method of fatty acid estimation, the procedure, noted in a preliminary report (5), is described here in detail.

## EXPERIMENTAL

### *Materials*

Ethyl ether, reagent grade.	NaOH, N/50 aqueous.
Na <sub>2</sub> SO <sub>4</sub> , granular.	Thymol blue, 0.1% alcoholic.
HCl, 1 N.	Sodium dodecyl sulfate, 5%.

<sup>1</sup> Tuberculosis Control Division, U. S. Public Health Service.

<sup>2</sup> Furnished through the courtesy of the Atlas Powder Co., Wilmington, Delaware.



*Extraction of Fatty Acid from Aqueous "Tween" Solution*

Emphasis was placed on developing a rapid, simple method for extracting small amounts of fatty acid from dilute solution; unnecessary manipulations, such as drying or filtration, were avoided. Table I briefly outlines the procedure and presents evidence of its efficiency. It is seen that extraction of oleic acid from aqueous solution is complete. The procedure described is sensitive to 0.005 ml. of *N*/50 NaOH, *i.e.*, 0.1 micromole of fatty acid, corresponding to 28  $\gamma$  of oleic acid. The various steps will be discussed in turn.

TABLE I

*Extraction of Oleic Acid from Aqueous Solution*

To test the completeness of the extraction, the direct titer of an alcoholic solution of oleic acid was compared with the titer of identical samples added to 15. ml. of *N*/100 HCl, saturated with  $\text{Na}_2\text{SO}_4$ , and extracted by three 2 ml. portions of ether. The extracts were evaporated as indicated. All samples were made up to a volume of 2 ml. with alcohol and titrated with *N*/50 aqueous NaOH to an olive green end point with thymol blue.

Sample	Ether extraction	Duplicate titer N/50 NaOH	
	ml.	ml.	
<i>Direct titration</i>			
1.00 ml. approx. 0.85% oleic acid in alcohol titrated directly	—	1.545	1.535
Same + 5 ml. ether, evaporated to dryness	—	1.545	1.55
Same + 5 ml. ether, evaporated to 1 ml.	—	1.54	1.545
<i>Extraction and titration</i>			
Blank extraction of 15 ml. N/100 HCl, sat. Na <sub>2</sub> SO <sub>4</sub>	6	0.02	0.015
Same + oleic acid, evaporated to dryness	3,2,2	1.56	1.555
Same + oleic acid, evaporated to 0.5 ml.	3,2,2	1.56	1.56

Saturation of the aqueous phase with an excess of solid  $\text{Na}_2\text{SO}_4$  (1 g./5 ml.) permits separation of the ether in the presence of "Tween" 80, which otherwise causes emulsification. Since strong acidification would be a source of error in the present procedure, through frequent accidental entrainment of tiny droplets of water in the ether, the solution is acidified with HCl to approximately pH 2 (*i.e.*, *N*/100), conveniently recognized by the salmon pink color of added thymol blue (one drop of 0.1% alcoholic solution). This operation is carried out before saturating with  $\text{Na}_2\text{SO}_4$ , which markedly shifts the color of the indicator. The ether subsequently extracts the indicator, the alkaline range of which permits it also to serve in the later titration of fatty acid. As is pointed out in a later section on extraction in the presence of serum albumin, long chain fatty acids may be extracted following buffering at a pH as high as 6.0–6.5.

In simple solutions of "Tween," however, it is preferable to use a lower pH, at which extraction is more efficient.

The ether is equilibrated with the aqueous solution by stirring in an open test tube, which has proved vastly superior to shaking in a tightly stoppered vessel. Efficient emulsification without spattering is produced by rapidly raising and lowering a long glass rod, with a tip flattened to a disc fitting the tube like a loose piston. The ether, after its separation as a layer, is carefully pipetted to a small test tube ( $12 \times 100$  mm.).

Moderate amounts of oleic acid are quantitatively extracted from 10–20 ml. of aqueous solution by three successive 2 ml. portions of ether (the first portion of ether being increased by an extra 1 ml./10 ml. of water to allow for saturation of the latter). Completeness of the extraction may be tested by separately titrating an additional extraction at the end of the regular series; if it has a higher titer than the blank, the original extraction was incomplete. In the presence of high concentrations of "Tween" (e.g.  $>1\%$ ), where extraction is somewhat less efficient, four portions of ether are used.

The combined portions of ether in the test tube, after addition of two or three pieces of washed Alundum, are evaporated by immersion in hot water in a hood. The less volatile final drop need not be evaporated completely, as shown in Table I, which also shows that added alcohol does not esterify the fatty acid.

The residue in the tube is dissolved by addition of 1.0 ml. of ethyl alcohol, and 1 drop of 0.1% thymol blue in alcohol is added unless it had been added to the aqueous solution before acidification. The solution is titrated to an olive green end point with  $N/50$  aqueous NaOH, with constant mixing by a bubbling stream of  $\text{CO}_2$ -free air. At this color, occurring at approximately pH 8, a sharp and stable end point can be obtained with a precision of 0.005 ml. A blank on 1 ml. of alcohol titrates 0.01–0.015 ml.; the acid residue of 6–8 ml. of ether of reagent or anesthetic grade is negligible.

Aqueous alkali, which has several advantages over sodium ethylate, has the disadvantage of precipitating fatty acid at high titers. In such circumstances additional alcohol may be added, with appropriate correction of the blank.

Table II shows that the extraction is efficient in the presence of "Tween" 80.

#### *Free Fatty Acid in "Tween"*

The titer of free fatty acid found in several lots of the commercial product "Tween" 80, 0.12 ml.  $N/50$  per 1 ml. of 10% solution (Table II), corresponds to 3% of the total fatty acid present, or 0.6% of the "Tween" 80 by weight, if the fatty acid is calculated as oleic acid. These calculations follow from the manufacturer's information that "Tween" 80 is a reaction product of 1 mole of sorbitol anhydride (average composition corresponding to sorbitan, the monoanhydride) per mole of oleic acid and per 20 moles of ethylene oxide; of this compound 22.5% should be recoverable as oleic acid. The esterified oleic

TABLE II

*Extraction of Oleic Acid from "Tween" 80*

The extractions were performed as in Table I, except that the same pair of tubes of *N*/100 HCl saturated with  $\text{Na}_2\text{SO}_4$  was used for all the successive additions and extractions. The initial blank is, as usual, slightly higher than blanks following exhaustive extraction. The difference represents traces of ether-soluble acid in the glassware, water, and  $\text{Na}_2\text{SO}_4$ . The low reextraction titers show complete extraction by three 2 ml. portions of ether. The titer of "Tween" 80 plus added oleic acid equals the sum of the two extracted separately, minus the extra blank; this indicates complete recovery in the presence of "Tween" 80.

Sample added to 15 ml. solution	Ether ml.	Titer <i>N</i> /50 NaOH Duplicate	
		ml.	'
Blank	6	0.02	0.02
1.00 ml. 0.5% oleic acid in alcohol	2,2,2	1.065	1.07
Reextract (blank)	6	0.02	0.015
Reextract (blank)	6	0.015	0.015
1.00 ml. 10% "Tween" 80	2,2,2	0.135	0.13
Remove "Tween" 80 at interface			
1.00 ml. 10% "Tween" 80 + 1.00 ml. 0.5% oleic acid	2,2,2	Accident	1.18

acid in "Tween" 80 was analytically determined by the method described here, following alkaline hydrolysis, and was found substantially to verify the theoretical composition. A method of preparing essentially fatty acid-free "Tween" 80 from the commercial product is described in the following paper (6).

The analytical method is equally applicable to esters of other long chain fatty acids. It was found, for example, that added stearic acid was quantitatively recovered from aqueous solutions of "Tween" 60, a stearic acid homologue of "Tween" 80, and that "Tween" 60 contained only 1/6 as much free fatty acid as "Tween" 80.

*Extraction in the Presence of Albumin*

Serum albumin emulsifies water in ether, despite saturation with  $\text{Na}_2\text{SO}_4$  or several other highly soluble salts. It was found that addition of sodium dodecyl sulfate ( $\text{C}_{12}\text{H}_{25}\text{SO}_4\text{Na}$ , commercially available under the trade mark "Duponol C") permits ether extraction in the presence

of albumin or of whole serum. The  $C_{12}H_{25}SO_4Na$  is added to produce a final concentration approximately half that of the albumin; if the ratio deviates much in either direction, emulsification occurs. Separation of the ether from albumin- $C_{12}H_{25}SO_4Na$  mixtures occasionally requires brief centrifugation.

A complication arises from the contamination of commercial albumin by sodium acetate, which, under the extraction conditions described above, causes the albumin solutions to continue to yield small amounts of titratable acid beyond the third or fourth portion of ether. The extraction of short chain water-soluble fatty acids, such as acetic acid, is minimized by buffering the solution at the highest pH (6.0) which permits quantitative extraction of the oleic acid. Accordingly, in the presence of albumin containing sodium acetate the aqueous solution is not acidified to pH 2, but is rather treated with 1/10 its volume of 0.2 *M* phosphate buffer at pH 6.0. It is then saturated with  $Na_2SO_4$  and extracted in the usual way. This modified method is still subject to slight error from lower fatty acids, and hence cannot be applied indiscriminately with complete confidence in its specificity. It is adequate, however, for the purpose for which it was developed, the detection of traces of lipase in serum albumin, since the error is small and constant in successive aliquots.

Table III demonstrates the complete recovery of added oleic acid in the presence of serum albumin and  $C_{12}H_{25}SO_4Na$  at pH 6. It also shows that the protein blank is much higher at pH 2 than at pH 6, and that the responsible substance is dialyzable and is not completely extracted by three portions of ether; these properties differentiate it from higher fatty acids. In addition, since the proportion of  $C_{12}H_{25}SO_4Na$  to protein seems to be less critical with serum than with albumin, it was possible to show with serum that the titer is independent of the amount of  $C_{12}H_{25}SO_4Na$  added.

This method offers the possibility of investigating the presence of free fatty acid in serum by a gentle method which avoids heating of the serum and its possible resultant hydrolysis. Preliminary investigation has shown in fresh normal human serum (Table III) approximately 0.02 ml. *N*/50 ether-soluble acid/ml. serum, *i.e.*, 0.4 mM., corresponding to 10 mg.-% if calculated as oleic acid. Although this amount is very small, it is considered to be genuine higher fatty acid, for it is completely extracted by the usual three portions of ether, and the titer is proportional to the amount of serum extracted. A specimen of serum from the same subject, stored four months in the refrigerator, contained approximately 8 times as much free fatty acid as the fresh serum, indicating that considerable lipolysis took place during storage.

TABLE III

*Extraction of Fatty Acid from Serum Albumin and from Serum*

Extractions as described in Table I were performed from 10 ml. of solution, except that, where indicated, instead of acidification to pH 2 the solution was buffered by addition of 1/10 its volume of 0.2 M phosphate buffer at pH 6.0.

Sample	Titer N/50 NaOH	
	Extraction 2.2,2 ml. ether	Reextraction 6 ml. ether
<i>Buffered at pH 6.0</i>		
Blank	0.015	—
Oleic acid, 1.0 ml. approx. 0.2%	0.35	0.02
Albumin blank (0.4 ml. 5% bovine albumin + 0.2 ml. 5% $C_{12}H_{25}SO_4Na$ )	0.025	—
Albumin + $C_{12}H_{25}SO_4Na$ + oleic acid	0.37	0.015
<i>Acidified to pH 2</i>		
Albumin blank	0.095	0.06
Same except albumin dialyzed overnight	0.045	0.025
<i>Serum buffered at pH 6.0</i>		
0.4 ml. stored human serum + 0.5 ml. 2% $C_{12}H_{25}SO_4Na$	0.08	0.02
0.4 ml. fresh human serum + 0.5 ml. 2% $C_{12}H_{25}SO_4Na$	0.025	0.015
2.0 ml. fresh human serum + 3.0 ml. 2% $C_{12}H_{25}SO_4Na$	0.05	0.02
2.0 ml. fresh human serum + 2.0 ml. 2% $C_{12}H_{25}SO_4Na$	0.05	0.02
2.0 ml. fresh human serum + 1.0 ml. 2% $C_{12}H_{25}SO_4Na$	0.055	0.02

## DISCUSSION

The simple and rapid method of extracting and titrating fatty acids described here is believed to involve smaller amounts of material than most methods which have previously been published, with the exception of the elegant micro method of Schmidt-Nielsen (7). In addition, it involves no complicated apparatus, and is readily adaptable to routine analysis. It is possible to analyze 6 samples completely in one hour. A related method for extracting fatty acids in the presence

of "Tween" 20, a lauryl ester, has been briefly described by Archibald (8).

While the method has been developed for the specialized purpose of estimating fatty acid in the presence of "Tween" or proteins, it appears to have some advantage over several available procedures for determining the fatty acid in lipid hydrolyzates. The method of Stewart and White (9), for example, involves addition of a measured amount of strong alkali for saponification, followed by back titration with acid, the fatty acid then being a small difference between two large quantities. The method of Stoddard and Drury (10, 11) involves collection on a filter paper of the fatty acid; this involves extra manipulations with possible loss, demands large amounts of material, and is inapplicable to those fatty acid mixtures which are liquid at room temperature.

The availability of a method for extracting free fatty acids in the presence of proteins permits more precise investigation of physiological problems involving fat transport: for example, the question whether fatty acids are entirely esterified before absorption from the intestinal epithelial cells into the blood or chyle (12).

#### ACKNOWLEDGMENT

The technical assistance of Mrs. Harlean Cort is gratefully acknowledged. The author is indebted to Drs. Jordi Folch, R. M. Archibald, and R. D. Hotchkiss, of the Rockefeller Institute for Medical Research, for valuable suggestions, and to Dr. R. J. Dubos for constant interest and encouragement.

#### SUMMARY

A simple, rapid ether extraction method is described for estimating higher fatty acids in the presence of "Tween," water-soluble esters of these acids. The method is sensitive to  $0.1 \mu M$  of fatty acid (28  $\gamma$  of oleic acid) and is suitable for extracting small amounts from large volumes of dilute solution.

With this method it was shown that 3% of the oleic acid in the commercial product "Tween" 80 is unesterified.

In the presence of serum proteins quantitative extraction of fatty acid is possible following the addition of sodium dodecyl sulfate. Small amounts of free fatty acid (0.4 mM) were found in fresh normal human serum.

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# The Preparation and Stability of Fatty Acid-Free Polyoxyethylene Sorbitan Monooleate ("Tween" 80)

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Received June 9, 1947

## INTRODUCTION

The preceding paper (1) described an analytical method for estimating small amounts of fatty acid in the presence of certain water-soluble esters of long-chain fatty acids (marketed under the trade mark "Tween" <sup>2</sup>). By this method 3% of the fatty acid was found to be unesterified in "Tween" 80, a standardized commercial polyoxyethylene derivative of sorbitan monooleate. This impurity is sufficient to account for the observed inhibitory effect of "Tween" 80 on the growth of small inocula of tubercle bacilli (2, 3, 4). Since only direct testing of fatty acid-free "Tween" 80, however, could prove that the ester itself is non-toxic, a method of purifying "Tween" was developed. Because biological applications of "Tween" 80 might possibly be extended by eliminating its free fatty acid, the procedure for removing this impurity is described here in detail. A somewhat similar method for purifying "Tween" 20 (a lauryl ester), in larger lots, has been described by Archibald (5).

## EXPERIMENTAL

### *Preparation and Estimation of Fatty Acid-Free "Tween" 80*

Fatty acid-free "Tween" 80 is conveniently prepared in small lots. For example, 2 g. of the commercial product is dissolved in 30 ml. of *N*/100 HCl in a large test tube

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<sup>1</sup> Tuberculosis Control Division, U. S. Public Health Service.

<sup>2</sup> Furnished through the courtesy of the Atlas Powder Company, Wilmington, Delaware.



and saturated with an excess of  $\text{Na}_2\text{SO}_4$  (approximately 6 g.). The mixture is extracted four times with 10 ml. portions of ether, intimately mixed with the aqueous solution by rapidly raising and lowering a glass rod with a tip flattened to a disc fitting the test tube like a loose piston. Brief centrifugation readily separates the ether layer, which is pipetted off and discarded.<sup>3</sup>

Following the final extraction, the tube is centrifuged vigorously and the supernatant gummy "Tween" 80 layer is removed with the aid of the glass rod and heated over a water bath until the residual ether has evaporated. There remains with the purified "Tween" 80 a small amount of dilute HCl saturated with  $\text{Na}_2\text{SO}_4$ . This is partly removed by strips of filter paper, but, since traces of  $\text{Na}_2\text{SO}_4$  were not significant in the bacteriological experiments for which this material was prepared, further purification has not generally been carried out. The material can be further purified by dialysis in concentrated solution against a large volume of distilled water overnight, with only moderate further loss (*ca.* 10%). The slow rate of dialysis of "Tween" 80 through cellophane is presumably due to micelle formation.

Since the recovery of purified "Tween" 80 is incomplete, it is necessary for precise work to determine the yield. It was found possible to estimate "Tween" quantitatively by determining the total fatty acid by the method described (1), following hydrolysis by boiling or autoclaving (120°C.) for 30 minutes in 0.4 *N* NaOH. Analysis of "Tween" 80 in this manner yielded a content of oleic acid which was very close to the theoretical 22.5% by weight.<sup>4</sup> Analysis of several batches of purified "Tween" 80 indicated a recovery of 60–70%.

Table I shows that the process of purification has removed approximately 90% of the free fatty acid originally present in the "Tween" 80, leaving a concentration (*ca.* 0.06% of the "Tween" by weight) too small to be estimated accurately. The yield of purified "Tween" 80 may also be estimated by dry weight determination following dialysis to remove the  $\text{Na}_2\text{SO}_4$ , but the dialysis causes some loss.

<sup>3</sup> The ether, on evaporation, yields a larger residue than can be accounted for by its content of fatty acid, indicating that a small amount of "Tween" 80 is removed by the ether. Since the synthesis of "Tween" is stated by the manufacturer to yield a mixture of molecules with varying proportions of sorbitan, fatty acid, and ethylene oxide, it is probable that the ether selectively removes fractions with a higher proportion of oleic acid to sorbitan than the 1:1 molar ratio which statistically characterizes the mixture.

<sup>4</sup> The manufacturer states that "Tween" 80 consists of a reaction product of 1 mole of sorbitol anhydride (average composition corresponding to sorbitan, the mono-anhydride) per mole of oleic acid and per 20 moles of ethylene oxide, the fatty acid radical being joined to the sorbitan by ester linkage and the ethylene oxide units joined to each other and to the sorbitan by ether linkage. The average composition of this product corresponds to a minimum molecular weight of 1254, of which 282, or 22.5%, should be recoverable as oleic acid, following hydrolysis.

TABLE I

*Purity and Recovery of Fatty Acid-Free "Tween" 80*

Two g. of "Tween" 80 were purified as described in the text, and dissolved in 20 ml. of H<sub>2</sub>O, which would have formed a 10% solution if recovery had been complete. A portion was hydrolyzed by boiling 30 minutes in 10 volumes of 0.4 *N* NaOH. Extractions were performed as in Table I of reference (1) after dilution to 15 ml. of solution adjusted to pH 2 and saturated with Na<sub>2</sub>SO<sub>4</sub>.

Sample	Titer <i>N</i> /50		
	Extraction 3.2 ml. ether	Reextraction 6 ml. ether	Corrected titer
<i>Total fatty acid</i>			
A. 0.40 ml. hydrolyzed 10% solution purified "Tween" 80	1.06	0.05	1.08
B. Duplicate	1.085	0.03	1.085
<i>Free fatty acid</i>			
C. 2.00 ml. unhydrolyzed 10% solution purified "Tween" 80	0.03	0.015	0.015
D. 1.00 ml. fresh 10% solution unpurified "Tween" 80	0.13	0.015	0.12
E. Duplicate	0.135	0.015	0.125

The total fatty acid of the hydrolyzed purified "Tween" 80 has a corrected titer (A, B) of 1.08 ml., estimated by combining the titers of the initial extractions and the reextractions and subtracting twice the true blank of 0.015. Since 0.40 ml. of 10% "Tween" 80 has a theoretical titer of 1.60 ml. *N*/50, the recovery is 69%, and the "10%" solution is actually 6.9%. The corrected titer of free fatty acid of 1.0 ml. of 10% solution of the unpurified "Tween" 80 (D, E), 0.12, is 3% of the theoretical total fatty acid (4.00 ml. *N*/50). Since the corrected titer of the free fatty acid of the purified "Tween" (C) is only 0.015, near the limit of sensitivity of the method, for 2.0 ml. of 6.9% solution, approximately 90% of the free fatty acid has been removed by the process of purification.

*Stability of "Tween" 80*

It was observed that solutions of "Tween" 80 which stood at room temperature for a number of weeks became more bacteriostatic, due to an increase in their content of free oleic acid. After the preparation of purified "Tween" 80, it appeared desirable to study this spontaneous hydrolysis more carefully to determine how long a preparation might be expected to remain essentially fatty acid-free.

A freshly prepared 6.2% solution of purified "Tween" 80 was autoclaved at 120°C. for 10 minutes to eliminate bacterial contaminants which might complicate the results by introducing enzymatic lipolysis. Portions of this sterile solution were stored in the refrigerator and in the incubator and a 1:10 dilution also in the incubator. Samples of 2 ml. of the concentrated, and 10 ml. of the dilute, solution were withdrawn with sterile precautions at intervals up to 10 weeks for estimation of free fatty acid. In addition, a similar batch of purified "Tween" 80 was stored in the incubator as obtained from the process of purification—*i.e.*, as a solid contaminated by acidified  $\text{Na}_2\text{SO}_4$  solution. At the end of the experiment the free and total fatty acid in this specimen was estimated.

TABLE II

*Spontaneous Hydrolysis of Purified "Tween" 80 during Storage*

Specimens of "fatty acid-free" "Tween" 80 (*i.e.*, <1% unesterified) were stored at different concentrations and temperatures. Estimates of less than 1% approach the limit of sensitivity of the method and hence are not precise. Estimates of 70 and 90% are also rough since the excessively large sample of fatty acid was incompletely extracted.

Conditions of storage		Percentage of fatty acid unesterified					
Concentration	Temperature	Before auto- claving	After auto- claving	Duration of storage			
				1 wk.	2 wks.	3 wks.	10 wks.
6.2%	5°C.	0.4	0.4	0.7	0.6	0.9	2.4
6.2%	37°C.			1.1	3.0	5.2	>70
0.6%	37°C.			2.6	6.8	11.2	>90
Solid	37°C.	Assume 0.4 <sup>a</sup>		—	—	—	5.0

<sup>a</sup> Since the sample stored as a solid was not dissolved until the end of the experiment, no aliquot could be taken for estimation of free fatty acid at the start of the experiment. It is, therefore, necessary to assume the same initial free fatty acid concentration for this sample as for the identically prepared batch used in the stored solutions.

The results are expressed in terms of the percentage of the total fatty acid which is unesterified. (It will be recalled from Table I that unpurified "Tween" 80 is 3% unesterified—*i.e.*, it has a titer of 0.12 ml. *N*/50 acid/1 ml. of 10% solution.) Table II shows that the hydrolysis of "Tween" 80 is roughly five times as rapid at 37°C. as at 5°C. during the early stages of hydrolysis. At refrigerator temperature a concentrated solution (6.2%) may be kept three weeks without reaching 1% hydrolysis, and 10 weeks without attaining the degree of hydrolysis (3.0%) found in unpurified "Tween" 80, while in the

incubator such a solution is nearly completely hydrolyzed (over 70%) in 10 weeks. The hydrolysis is approximately twice as rapid in the dilute (0.6%) solution. The solid purified "Tween" 80 at incubator temperature, on the other hand, hydrolyzes so very much more slowly than the solutions that it seems reasonable to expect that solid purified "Tween" 80 could be stored for 2-3 months at refrigerator temperature without exceeding 1% hydrolysis.

It has been repeatedly observed that autoclaving at 120°C. for 10 minutes produces no perceptible hydrolysis of 10% "Tween" 80. This observation is of importance for the practical use of this product in bacteriological media.

### DISCUSSION

The process of purification described here eliminates 80-90% of the unesterified fatty acid in the commercial product "Tween" 80, reducing the concentration of this impurity to less than 0.1% by weight. The purified material was found to have no inhibitory effect on the growth of tubercle bacilli (3, 4). Indeed, since the purified "Tween" 80 itself protects tubercle bacilli against small amounts of added oleic acid, the trace of oleic acid remaining in the "fatty acid-free" "Tween" 80 is not inhibitory even when concentrations of "Tween" 80 as high as 0.8% are used in the culture medium (4).

The usefulness of fatty acid-free "Tween" 80 is not limited to cultivation of tubercle bacilli. For example, Clarke (6) has found that removal of free fatty acid by this method eliminates its inhibitory effect on the respiration of red blood cells containing malarial parasites. Fatty acid-free "Tween" 80 may have a variety of biological applications. Of particular interest is the possibility of intravenous alimentation, in which the free oleic acid might well be hemolytic.

### ACKNOWLEDGMENT

The technical assistance of Mrs. Harlean Cort is gratefully acknowledged. The author is indebted to Dr. R. J. Dubos for constant interest and encouragement.

### SUMMARY

A method is described for eliminating the free fatty acid which forms 3% of the total fatty acid of the commercial product "Tween" 80, and which causes it to be bacteriostatic to tubercle bacilli. Analysis

of the free and total fatty acid indicated a 70% yield, with elimination of approximately 90% of the impurity.

The rate of hydrolysis of "Tween" 80 in aqueous solution is briefly described. The purified product may be stored in concentrated solution in the refrigerator for several weeks without exceeding 1% hydrolysis, and in the solid form for much longer periods.

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# Comparative Effects of Liver and Yeast on Growth and Length of Survival of the Immature Thyroid-Fed Rat \*

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Received June 30, 1947

## INTRODUCTION

Available data indicate that, in addition to the major nutrients, substances are present in our diet which may be required in increased amounts during conditions of stress. Such factors are apparently dispensable under normal conditions, or their requirements are so small they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animals' own tissues. Certain drugs or other "stress factors" may, however, increase requirements for these substances to the extent that deficiencies occur, manifest by retarded growth or tissue pathology, and preventable by the administration in appropriate amounts of the missing nutrient. In the present series of experiments a condition of stress was induced by excessive thyroid feeding. Under such circumstances body requirements in the rat were increased for at least one unknown nutrient present in liver and required for normal growth and survival in the immature thyroid-fed rat.

## EXPERIMENTAL

### *No. 1. Comparative Length of Survival of Hyperthyroid Rats Fed Purified Rations Containing Synthetic Vitamins or Yeast as a Source of the B Complex Vitamins*

That hyperthyroidism results in an increased vitamin B requirement has been demonstrated by a number of investigators. Administration of thyroxin or thyroid

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\* The research which this paper reports was undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The views or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

gland to adult rats results in a prompt loss of weight preventable by administration of yeast or other concentrates of vitamin B (1, 2). In 1938 Drill and associates initiated a series of experiments on the effects of vitamin B<sub>1</sub> and yeast on adult hyperthyroid rats that had lost weight. It was observed that vitamin B<sub>1</sub> alone prevented further loss in weight, but that rats did not regain their initial weight unless a rich source of the vitamin B complex was also supplied (2, 3). Subsequent work demonstrated that pyridoxine and calcium pantothenate could effectively replace the vitamin B complex in the diet of the hyperthyroid rat (4). Thus, in addition to vitamin B<sub>1</sub>, both pyridoxine and pantothenic acid are required in larger amounts during experimental hyperthyroidism in the rat. Available data from this laboratory indicate, however, that other nutrients may be similarly involved. Thus Ershoff and Hershberg (5) found that length of survival was significantly greater in hyperthyroid rats fed yeast than in those receiving a similar amount of synthetic vitamins as a source of the B complex. The present experiment was undertaken to confirm and extend this observation.

Three basal diets were employed: diets A, B and C. Diets A and B were purified rations containing the B complex factors in synthetic form and differing only in their content of nicotinic acid. Diet C was similar in composition but contained yeast in place of the synthetic B factors. The synthetic vitamins were incorporated in diets A and B in amounts corresponding to their content in yeast so that the thiamine, riboflavin, pyridoxine and pantothenic acid content of the three diets was virtually identical. All three rations were supplemented with 0.0 and 0.5% U.S.P. desiccated thyroid. Since nicotinic acid was omitted from the synthetic rations employed in the earlier studies with thyroid-containing rations (5), it was incorporated in diet B in amounts corresponding to its content in the yeast-containing diet C, in order to rule out this vitamin as the factor in yeast responsible for prolonged survival.

Two experimental groups were employed consisting of male and female rats of the University of Southern California strain (Series I) and female rats of the Long-Evans strain (Series II). In Series I, animals were selected at 27-30 days of age and an average weight of 55 g.; in Series II, animals ranged from 21 to 23 days of age at the start of the experiment and averaged approximately 45 g. in weight. Litter mates were divided as far as possible among the 6 experimental groups listed in Table I. Animals were kept in metal cages with raised screen bottoms to prevent access to feces, and sufficient food was administered to assure *ad libitum* feeding. Feeding was continued for 168 days or until death, whichever occurred sooner.

Survival times are summarized in Table II. Length of survival of thyroid-fed rats was significantly longer in all cases for animals fed yeast than for those receiving the synthetic B vitamins. These differences were particularly marked in animals of the U.S.C. strain. Since both the synthetic and yeast-containing rations contained equal amounts of thiamine, riboflavin, pyridoxine and pantothenic acid, it is apparent that the beneficial effect of yeast on the survival time of thyroid-fed rats was not due to its content of the above nutrients. Furthermore, since no significant difference in length of survival was observed in hyperthyroid rats fed the two synthetic diets, and since

TABLE I  
*Composition of Experimental Diets<sup>1</sup>*

Dietary component	Diet A <sub>1</sub> and A <sub>2</sub>	Diet B <sub>1</sub> and B <sub>2</sub>	Diet C <sub>1</sub> and C <sub>2</sub>
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Yeast <sup>2</sup>	0.0	0.0	12.0
Vitamin test casein <sup>3</sup>	22.0	22.0	22.0
Salt mixture <sup>4</sup>	4.5	4.5	4.5
Sucrose	73.5	73.5	61.5

Vitamin Supplements Added to Diets

	<i>mg.-%</i>	<i>mg.-%</i>	<i>mg.-%</i>
Thiamine hydrochloride	7.2	7.2	0.0
Riboflavin	0.9	0.9	0.0
Pyridoxine hydrochloride	1.5	1.5	0.0
Calcium pantothenate	6.72	6.72	0.0
Nicotinic acid	0.0	6.0	0.0
2-Methylnaphthoquinone	0.5	0.5	0.5
Choline chloride	120.0	120.0	120.0

U.S.P. desiccated thyroid (Armour) was incorporated in diets A<sub>2</sub>, B<sub>2</sub> and C<sub>2</sub> at a level of 0.5%, replacing an equal amount of sucrose.

<sup>1</sup> The following supplement was fed three times weekly to each rat: cottonseed oil (Wesson) 500 mg.,  $\alpha$ -tocopherol 1 mg., and a vitamin A-D concentrate<sup>5</sup> containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.

<sup>2</sup> Brewers' type yeast No. 200, Anheuser-Busch, Inc., St. Louis, Mo. Each gram, according to the manufacturer, contained the following vitamin potencies: thiamine 600  $\gamma$ , riboflavin 75  $\gamma$ , pyridoxine 100–125  $\gamma$ , pantothenic acid 420–560  $\gamma$ , and nicotinic acid 350–500  $\gamma$ .

<sup>3</sup> Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

<sup>4</sup> Salt Mixture No. 1 (Sure, B. (6)).

<sup>5</sup> Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D/g.

diet B contained an amount of nicotinic acid similar to that present in diet C, it is apparent that the beneficial effects of yeast were not due to its content of nicotinic acid. These results indicate that yeast contains some factor other than the above nutrients which prolonged survival of thyroid-fed rats under the conditions of the present experiment. Male rats appeared to be more susceptible to the effects of thyroid feeding than females. All animals fed the thyroid-free rations survived the experimental period of 168 days (6 animals per group).

The immediate cause of death was apparently heart failure. In



TABLE II  
*Comparative Length of Survival of Hyperthyroid Rats Fed  
 Purified Rations Containing Synthetic Vitamins or Yeast  
 as a Source of the B Complex Vitamins*

Experimental diet	Number of animals	Sex	Initial body weight	Average length of survival <sup>1,2</sup>	Range of survival <sup>2</sup>
Series I—U.S.C. Strain					
A <sub>2</sub>	12	F	54.9	29.6 ± 4.2	16– 60
B <sub>2</sub>	12	F	55.1	25.6 ± 4.7	12– 77
C <sub>2</sub>	12	F	54.9	91.8 ± 15.5	13–168
A <sub>2</sub>	10	M	53.3	19.3 ± 0.9	14– 24
B <sub>2</sub>	10	M	53.4	19.5 ± 1.0	14– 24
C <sub>2</sub>	10	M	53.2	41.7 ± 11.9	9–117
Series II—Long-Evans Strain					
A <sub>2</sub>	10	F	45.2	51.7 ± 12.0	13–132
B <sub>2</sub>	20	F	44.5	52.4 ± 11.1	14–158
C <sub>2</sub>	20	F	44.7	101.2 ± 16.1	24–168

<sup>1</sup> Including standard error of the mean calculated as follows:  $\sqrt{\frac{ed^2}{n}} / \sqrt{n}$  where  $d$  is the deviation from the mean and  $n$  is the number of observations.

<sup>2</sup> The experiment was terminated on the 168th day of feeding. At this time 7 female rats were still alive; 2 on diet C<sub>2</sub>, series I, and 5 on diet C<sub>2</sub>, series II. Averages were computed on the basis of a 168-day survival time for animals alive at the termination of the experiment.

agreement with earlier findings (5), ventricles were invariably found at autopsy in a state of contraction, with the auricles and pulmonary vein engorged with blood. Heart weights were increased and the ventricular wall was hypertrophied, but otherwise, with the exception of infantile-like ovaries, the gross appearance of thyroid-fed rats was essentially normal. Chemically, a marked reduction was observed in the creatine content of the heart. In agreement with earlier findings (7), the concentration of ventricular creatine in thyroid-fed rats averaged at death approximately 50% of that obtained in animals fed similar rations with thyroid omitted. No significant difference in creatine concentration was observed between animals fed the synthetic

and yeast-containing diets.<sup>1</sup> Growth was retarded on all thyroid-containing diets; but animals grew consistently on these rations until a few days before death. In general, animals plateaued or lost weight before dying although, in some cases, if subjected to excitation, they succumbed before a growth plateau had been reached. The impression was gained that thyroid-fed rats on synthetic rations were more likely to succumb following excitation than those on the yeast-containing ration. In preliminary work it was observed that merely transferring thyroid-fed rats from one cage to another would result in some cases in sufficient excitation to cause death in a matter of a few minutes. Accordingly, in the present experiment handling was reduced to a minimum, and animals were not removed from their cages except for weekly weighings. In all dietary groups thyroid-fed rats developed some diarrhea during the first month of feeding, and their fur invariably became dull, rough and dirty. This was particularly marked in animals fed the synthetic diets. In the absence of dietary thyroid no significant differences in growth or gross appearance were observed on any of the rations, and the fur remained clean and sleek.

*No. 2. Effects of Dietary Supplements on Growth and Length of Survival of Thyroid-Fed Rats*

Available data indicate that toxic doses of thyroid result, in the young rat, in a marked retardation of growth. In the adult rat, loss of weight following excessive thyroid administration may be prevented by increased amounts of thiamine or yeast (2, 3); or, if weight were already lost, initial weight might be regained following increased dosage with thiamine, pyridoxine and calcium pantothenate (4). Such nutrients were without effect, however, on the growth rate of young thyroid-fed rats (5). Since retardation of growth is commonly observed in animals suffering from nutritional deficiency, it was felt that excessive thyroid feeding may have precipitated in the young rat a deficiency of some nutrient other than the above necessary for optimal growth. Accordingly, attempts were made to prevent by dietary means the retardation of growth of the immature thyroid-fed rat.

<sup>1</sup> We are indebted to Mrs. Virginia Hughes Brunish for all chemical determinations. Total tissue creatinine was determined by the method of Rose, Helmer and Chanutin (8) with results expressed as creatine. Subsequent findings indicate that the addition of 10% liver or 0.5 and 1.0% creatine to diet B<sub>2</sub> was without significant effect on the concentration of ventricular creatine in animals dying in the course of the experiment.

The experimental rations employed in the present experiment consisted of diet B<sub>2</sub> and similar rations containing, respectively, 10% yeast,<sup>2</sup> wheat germ,<sup>3</sup> whole liver powder,<sup>4</sup> pancreas,<sup>5</sup> or casein,<sup>6</sup> added to diet B<sub>2</sub> in place of an equal amount of sucrose. Female rats of the Long-Evans strain were weaned at 21-23 days of age and fed *ad libitum* the diets listed above. Feeding was continued for 100 days or until death, whichever occurred sooner.

Results of the present and subsequent experiments are summarized in Table III. A significant difference in growth and length of survival was observed on the various diets employed. Survival times were significantly greater on yeast- or liver-containing rations than on other diets tested, while liver completely counteracted the retardation of growth observed on all other thyroid-containing rations. No significant differences in growth or length of survival were observed between animals fed diet B<sub>2</sub> and those on similar rations containing wheat germ or casein, while the shortest of all survival times occurred on the pancreas-containing ration.

Subsequent experiments were conducted on the effects of each of the individual vitamins on growth and length of survival of the immature thyroid-fed rat. The respective vitamins were incorporated in diet B<sub>2</sub> in the following amounts/kg. of ration: thiamine hydrochloride 3.6 g., riboflavin 450 mg., pyridoxine hydrochloride 750 mg., calcium pantothenate 3.36 g., nicotinic acid 3.0 g., inositol 10.0 g., *p*-aminobenzoic acid 10.0 g., biotin 1.0 mg., folic acid 10.0 mg., ascorbic acid 10.0 g., vitamin A 100,000 U.S.P. units, vitamin D 25,000 U.S.P. units and 2-methylnaphthoquinone 100 mg.  $\alpha$ -Tocopherol was tested as a daily supplement at a level of 5 mg. per rat. Female rats of the Long-Evans strain were weaned at 21-23 days of age and fed each of the above diets *ad libitum* for 100 days or until death, whichever occurred sooner. Similar experiments were also conducted with the following supplements: (1) 0.3 and 0.6% DL-methionine, (2) 0.5 and 1.0% creatine, (3) 4.5% salt mixture in addition to that present in diet B, and (4) inositol plus *p*-aminobenzoic acid in the amounts indicated above.

Results are summarized in Table III. No significant differences in growth or length of survival were observed in the immature thyroid-fed rat on any of the above rations. Only liver and yeast of all substances tested prolonged survival, and only liver caused a significant increase in rate of growth. The beneficial effects of liver were similarly demonstrated in respect to ovarian weight and appearance. In all thyroid-fed

<sup>2</sup> Brewers' type yeast No. 200, Anheuser-Busch, Inc., St. Louis, Mo.

<sup>3</sup> Defatted Wheat Germ, Vio-bin Corporation, Monticello, Ill.

<sup>4</sup> Whole Dried Liver Powder, Armour and Co., Chicago, Ill.

<sup>5</sup> Pancreas Substance Powder, Armour and Co., Chicago, Ill.

<sup>6</sup> Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

rats with the exception of those receiving liver, gonadal development was inhibited, with ovaries resembling in weight and microscopic appearance those of the immature rat. These effects were completely counteracted by liver feeding, with ovaries in this group resembling in all respects those of the normal rat (9).

Available data indicate that the factor or factors in liver responsible for the above effects are heat-stable. Autoclaving liver at 15 lbs.-pressure for 1 hour and 45 minutes did not alter its effectiveness in the immature thyroid-fed rat (Table III). Subsequent work indicates the active principle is either water-insoluble or is chemically bound so that it may not readily be removed by simple water extraction.

Preliminary experiments conducted with Liver Concentrate Powder 1-20 (Wilson)<sup>7</sup> added to diet B<sub>2</sub> at a level of 4% of the diet, indicate that this preparation, which contains the water-extractable material of raw liver, had virtually no activity in respect to prolonging survival or promoting growth in the immature thyroid-fed rat. On the other hand, Extracted Liver Residue (Wilson) consisting of the coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances, was as effective at a 10% level as whole liver powder in promoting growth and prolonging survival in the immature thyroid-fed rat (Table 3).

### *No. 3. Comparative Effects of Liver and Yeast on Appetite and Efficiency of Food Utilization in the Immature Thyroid-Fed Rat*

Data reported above indicate that liver completely counteracted the retardation of growth observed on all other rations containing similar amounts of thyroid. This effect may have occurred through any one or more of the following processes: (1) an increased food consumption; (2) an increased efficiency of food utilization; or (3) neutralization, elimination or destruction of the thyroid hormone. The purpose of the present experiment was to determine the comparative effects of liver and yeast on food consumption and efficiency of food utilization in the immature thyroid-fed rat.

Female rats of the Long-Evans strain were weaned at 21-23 days and fed *ad lib.* the following three rations: (1) diet B<sub>2</sub>, (2) diet B<sub>2</sub> plus 10% yeast, and (3) diet B<sub>2</sub> plus 10% whole liver powder, with the liver and yeast replacing an equal amount of sucrose. Animals were placed in individual metal cages with raised screen bottoms to prevent access to feces, and food and water intake was determined daily for 28 days. Only animals surviving the 28-day feeding period were considered in the tabulation of data.

<sup>7</sup> We are indebted to Dr. David Klein of the Wilson Laboratories, Chicago, Ill., for the Liver Concentrate Powder 1-20 and the Extracted Liver Residue employed in the present experiment.

TABLE III  
*Effects of Dietary Supplements on Growth and Length of  
 Survival of Thyroid-Fed Rats*

Experimental diet	Num- ber of animals	Initial body weight	Average gain in body weight on following days of expt.			Per cent surviv- ing <sup>1</sup>	Average survival time <sup>2, 3</sup>
			28th	60th	100th		
B <sub>2</sub>	30	g. 44.5	g. 40.3 (22)	g. 64.2 (4)	g. 96.0 (1)	3.3	days 43.1 ± 9.2
B <sub>2</sub> and ThiamineHCl	10	44.0	40.5 (4)	66.8 (3)	78.0 (1)	10.0	41.4 ± 9.4
B <sub>2</sub> and Riboflavin	10	44.1	35.7 (7)	69.3 (3)	84.5 (2)	20.0	53.5 ± 9.1
B <sub>2</sub> and Pyridoxine HCl	10	44.1	44.5 (6)	50.0 (2)	76.0 (1)	10.0	41.7 ± 7.3
B <sub>2</sub> and Ca Pantothenate	10	45.6	44.4 (6)	73.6 (3)	—	0.0	42.9 ± 8.6
B <sub>2</sub> and Nicotinic acid	10	45.7	49.5 (4)	75.5 (2)	100.0 (1)	10.0	39.1 ± 8.4
B <sub>2</sub> and <i>p</i> -Aminobenzoic acid	10	44.2	32.6 (5)	81.5 (2)	—	0.0	40.1 ± 9.5
B <sub>2</sub> and Inositol	10	44.0	49.3 (9)	86.8 (5)	115.0 (1)	10.0	58.9 ± 9.0
B <sub>2</sub> and PAB and Inositol	10	44.2	46.6 (5)	82.3 (3)	113.0 (1)	10.0	41.6 ± 8.6
B <sub>2</sub> and Folic acid	10	45.7	57.7 (6)	91.5 (2)	—	0.0	39.2 ± 9.7
B <sub>2</sub> and Biotin	10	44.3	61.0 (8)	87.3 (4)	137.0 (1)	10.0	56.1 ± 7.8
B <sub>2</sub> and Ascorbic acid	10	40.5	62.3 (8)	—	—	0.0	34.3 ± 3.0
B <sub>2</sub> and Vitamin A	10	40.5	60.0 (6)	126.0 (1)	—	0.0	32.0 ± 5.4
B <sub>2</sub> and Vitamin D	10	40.5	53.4 (6)	—	—	0.0	33.1 ± 3.6

The values in parentheses indicate the number of animals that survived of which this is an average.

<sup>1</sup> Experimental period—100 days.

<sup>2</sup> Averages were computed on the basis of a 100-day survival time for animals alive at the termination of the experiment.

<sup>3</sup> Including standard error of the mean.

TABLE III—*Continued*

Experimental diet	Number of animals	Initial body weight	Average gain in body weight on following days of expt.			Per cent surviving <sup>1</sup>	Average survival time <sup>2,3</sup>
			28th	60th	100th		
B <sub>2</sub> and $\alpha$ -Tocopherol	10	g. 40.1	g. 61.4 (9)	g. 88.0 (4)	g. —	0.0	days 53.8 $\pm$ 5.1
B <sub>2</sub> and 2-Methylnaphthoquinone	10	40.4	61.3 (9)	99.0 (4)	—	0.0	49.5 $\pm$ 5.9
B <sub>2</sub> and 0.3% DL-Methionine	10	44.0	45.4 (9)	—	—	0.0	41.0 $\pm$ 3.5
B <sub>2</sub> and 0.6% DL-Methionine	10	44.1	33.1 (9)	76.0 (2)	—	0.0	47.7 $\pm$ 4.7
B <sub>2</sub> and 0.5% Creatine	10	44.5	56.4 (8)	71.0 (2)	—	0.0	49.8 $\pm$ 6.1
B <sub>2</sub> and 1.0% Creatine	10	43.6	54.5 (6)	90.5 (2)	—	0.0	46.5 $\pm$ 7.8
B <sub>2</sub> and Salt mixture	10	44.5	40.2 (8)	42.0 (2)	68.0 (1)	10.0	50.1 $\pm$ 7.8
B <sub>2</sub> and 10% Casein	10	43.8	59.0 (6)	—	—	0.0	35.0 $\pm$ 3.4
B <sub>2</sub> and 10% Wheat germ	10	45.3	75.0 (9)	90.5 (2)	99.0 (1)	10.0	51.4 $\pm$ 7.9
B <sub>2</sub> and 10% Pancreas	10	43.3	40.0 (1)	—	—	0.0	20.6 $\pm$ 2.2
B <sub>2</sub> and 10% Yeast	20	44.7	71.7 (17)	90.3 (13)	106.0 (11)	55.0	76.0 $\pm$ 8.7
B <sub>2</sub> and 10% Whole liver powder	20	44.5	103.2 (17)	155.5 (15)	175.0 (12)	60.0	78.1 $\pm$ 9.9
B <sub>2</sub> and 10% Autoclaved whole liver powder	10	45.0	86.8 (10)	136.3 (9)	175.4 (7)	70.0	91.9 $\pm$ 5.0
B <sub>2</sub> and 4% Liver Concentrate 1:20 (Wilson)	10	42.1	68.5 (8)	124.0 (1)	—	0.0	39.6 $\pm$ 5.1
B <sub>2</sub> and 10% Extracted Liver Residue (Wilson)	10	41.6	113.1 (10)	162.4 (9)	183.2 (5)	50.0	86.3 $\pm$ 4.9
B <sub>1</sub>	10	42.7	102.3 (10)	156.0 (10)	177.8 (10)	100.0	100.0 $\pm$ 0.0
B <sub>1</sub> and 10% Yeast	10	43.1	108.8 (10)	159.7 (10)	186.5 (10)	100.0	100.0 $\pm$ 0.0
B <sub>1</sub> and 10% Whole liver powder	10	42.7	116.4 (10)	168.3 (10)	189.2 (10)	100.0	100.0 $\pm$ 0.0

Results are summarized in Table IV. Food consumption for animals fed liver was greater than for animals fed the synthetic or yeast-containing rations. The increased food consumption of the liver-fed rats was noted as early as the fifth day of feeding and remained consistently greater for the duration of the experiment. These findings indicate that the increased growth of the immature thyroid-fed rat receiving liver was correlated with, if not entirely due to, an increased food consumption. In the absence of dietary thyroid no significant differences in rate of growth were noted on any of the above diets (Table III) nor were differences noted in total food consumption. These findings indicate that the increased growth and food consumption of thyroid-fed rats receiving liver were due to a factor in liver not present in significant amounts in the other diets employed, but required in increased amounts by the hyperthyroid rat. Available data indicate that efficiency of food utilization as measured by gain in weight/g. food ingested was greater in thyroid-fed rats receiving liver than for animals fed the synthetic or yeast-containing rations. Further experiments, however, involving studies with techniques such as the paired-feeding method are indicated for proper evaluation of these data.

TABLE IV  
*Comparative Effects of Liver and Yeast on Food and Water Intake and Efficiency of Food Utilization in the Immature Thyroid-Fed Rat*

Dietary group	Number of animals	Initial body weight	Gain in body weight for 28-day period	Average daily intake per rat during following weeks of experiment								Total food intake per rat for 28-day period <sup>1</sup>	Average gain in weight/g. food eaten.
				Food <sup>1</sup>				Water					
				1st	2nd	3rd	4th	1st	2nd	3rd	4th		
		<i>g.</i>	<i>g.</i>	<i>g./day</i>				<i>cc./day</i>				<i>g.</i>	<i>g.</i>
B <sub>2</sub>	8	45.1	39.9	7.5	12.5	13.1	13.7	9.5	12.4	14.1	15.5	327.1	.12
B <sub>2</sub> +Yeast	9	44.9	65.8	8.1	13.7	14.6	16.0	10.4	16.0	18.7	19.3	366.0	.18
B <sub>2</sub> +Liver	9	45.1	101.1	8.5	15.2	18.2	17.8	12.9	19.2	26.7	24.4	417.3	.27

<sup>1</sup> These values represent basal ration only and are exclusive of cottonseed oil ingested in supplementary feedings.

#### *No. 4. Comparative Effects of Liver and Yeast on the Basal Metabolism of the Immature Thyroid-Fed Rat*

The purpose of the present experiment was to determine the comparative effects of liver and yeast on the basal metabolism of immature thyroid-fed rats. It was felt that if the increased growth of liver-treated

rats was due to neutralization, elimination or destruction of the thyroid hormone, the basal metabolism of these animals would more closely approximate that of the normal animal than would readings obtained from thyroid-fed rats whose growth was retarded.

Accordingly, female rats of the Long-Evans strain were weaned at 21-23 days and fed *ad lib.* the following four diets: (1) diet B<sub>2</sub> plus 10% yeast, (2) diet B<sub>2</sub> plus 10% whole liver powder, (3) diet B<sub>1</sub> plus 10% yeast, and (4) diet B<sub>1</sub> plus 10% whole liver powder. The liver and yeast were added in place of an equal amount of sucrose. After six weeks of feeding, basal metabolic rates were determined for all groups.

We are indebted to Mr. G. D. Mason and Dr. R. J. Wenzler of the Department of Biochemistry and Nutrition, University of Southern California, for the B.M.R. determinations. The apparatus used was a closed circuit type with a capacity of two liters (10). Carbon dioxide was absorbed with sodium hydroxide, and oxygen consumption was determined from pressure changes recorded by means of a water manometer. The respiration chambers were kept at 28°C. and readings obtained were corrected to S.T.P. Food was removed from the animals' cages the evening prior to the metabolism test. At least 6 successive 5-minute intervals were recorded for each animal, with care being taken to record oxygen consumption when animal activity was at a minimum.

Findings are summarized in Table V. A significant increase in B.M.R. was observed in all rats fed thyroid. No significant difference in basal metabolic rate was observed, however, between liver and yeast-fed rats either (1) under conditions of thyroid-feeding or (2) on thyroid-free rations. Since the basal metabolism of thyroid-fed rats receiving liver did not differ significantly from that of thyroid-fed rats receiving yeast, although body weight was significantly greater in the former, it is concluded that the increased growth of the liver-fed rats was not due to neutralization, elimination or destruction of the thyroid hormone.

TABLE V  
*Comparative Effects of Liver and Yeast on the Basal  
Metabolism of Thyroid-Fed Rats*

Dietary group	Per cent thyroid	Number of animals	Oxygen consumption <sup>1</sup> cc./hr./100 g.	Change in B.M.R. <sup>2</sup> Per cent
B <sub>1</sub> +Yeast	0.0	9	136 ± 5.0	—
B <sub>2</sub> +Yeast	0.5	8	301 ± 10.7	+121
B <sub>1</sub> +Liver	0.0	10	127 ± 4.2	—
B <sub>2</sub> +Liver	0.5	12	280 ± 9.7	+120

<sup>1</sup> Including standard error of the mean.

<sup>2</sup> Compared to control B.M.R.



## DISCUSSION

Nutritional deficiencies have been induced in experimental animals by (1) diets deficient in essential nutrients, (2) bacteriostatic agents which interfered with the intestinal synthesis of essential nutrients, (3) inhibitory structural analogues which impaired the utilization of essential nutrients, and (4) drugs or other "stress factors" which increased metabolic requirements for essential nutrients on the part of the tissue cell (11). In the present experiment a deficiency state was induced by the latter method. Results indicate that excessive doses of thyroid increased requirements for at least one unknown nutrient in the immature female rat. Failure to provide this factor in adequate amounts resulted in deficiency symptoms, manifest in the thyroid-fed rat by retarded growth, inhibition of ovarian development and early death, apparently due to cardiac failure. The administration of whole liver powder completely counteracted the retardation of growth and inhibition of ovarian development observed above, while both liver and yeast prolonged significantly average survival on thyroid-containing rations. Available data indicate that the protective factor or factors is distinct from any of the known vitamins, either fat-soluble or water-soluble. Individual supplements of thiamine, riboflavin, pyridoxine, calcium pantothenate, nicotinic acid, inositol, *p*-aminobenzoic acid, biotin, folic acid or ascorbic acid were without effect on the above symptoms. Similar results were obtained with vitamins A and D,  $\alpha$ -tocopherol, 2-methylnaphthoquinone, creatine, DL-methionine, wheat germ or increased amounts of salt mixture or casein added at the expense of an equal amount of sucrose. Only liver and yeast of all substances tested prolonged survival, while only liver counteracted the retardation of growth and inhibition of ovarian development observed on all other thyroid-containing rations. Since liver and yeast both prolonged survival, while only liver increased growth in the immature thyroid-fed rat, the question arises whether the increased growth of liver-fed rats was due to a separate factor from that present in yeast or whether it might not reflect a higher concentration of the same factor concerned with prolonged survival. Insufficient data are now available to settle this point. Further work with more potent concentrates of active material or the use of liver at lower levels of feeding may help clarify this relationship.

Since no significant differences in growth, gross appearance, gonadal development or length of survival were noted on the various experi-

mental diets in the absence of dietary thyroid, it is apparent that the beneficial effects of liver and yeast in the thyroid-fed rat were due to the presence of some nutrient(s) in these substances whose requirement was increased as a result of thyroid-feeding and which was not provided in adequate amounts in other rations tested. The beneficial effects of liver on growth and ovarian development in the immature thyroid-fed rat were not due to neutralization, elimination or destruction of the thyroid hormone, since the basal metabolism of thyroid-fed rats receiving liver did not differ significantly from that of thyroid-fed rats receiving yeast although body and gonadal weight was significantly greater in the former. It is conceivable, however, that the thyroid preparation fed contained noxious material other than the hormone which was detoxified subsequent to liver feeding. This point might be clarified by repeating the experiment with thyroxin in place of the desiccated thyroid gland. Available data indicate that the beneficial effects of liver on the growth of immature thyroid-fed rats were due, at least in part, to a favorable influence on food consumption and efficiency of food utilization.

Findings indicate that the retardation of growth and inhibition of ovarian development observed in the immature thyroid-fed rat reflect a nutritional deficiency and are not specific effects of an excess of thyroid hormone *per se*. The mechanism by which thyroid hormone produced this deficiency is not clear. It is not unlikely, however, that increased metabolism following thyroid feeding may have accounted, at least in part, for the observed effects.

#### SUMMARY

Toxic doses of thyroid increased requirements for at least one unknown nutrient in the immature female rat. Failure to provide this factor in adequate amounts resulted in deficiency symptoms, manifest in the thyroid-fed rat by retarded growth and early death, apparently due to cardiac failure.

Whole liver completely counteracted the above retardation of growth, while both whole liver and yeast prolonged significantly average survival on thyroid-containing rations.

The protective factor(s) in whole liver and yeast was distinct from any of the known vitamins, either fat-soluble or water-soluble. Individual supplements of thiamine, riboflavin, pyridoxine, calcium pantothenate, nicotinic acid, inositol, *p*-aminobenzoic acid, biotin, folic acid

or ascorbic acid, were without effect on the above symptoms. Similar results were obtained with vitamins A and D,  $\alpha$ -tocopherol and 2-methylnaphthoquinone. Increasing the salt and casein content of the experimental rations was also without effect.

The beneficial effects of whole liver on the growth of immature thyroid-fed rats were correlated with increased food consumption and an increased efficiency of food utilization.

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# Effect of Growth Substrate on the Enzymic Constitution of *Aerobacter indologenes*\*

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Received February 21, 1947

## INTRODUCTION

Since the extensive investigations of Karström (4) on enzymic variation, this phenomenon has received increasing attention. Stephenson (11) divided variations into two main groups, those genetically impressed, resulting in selective multiplication of normally occurring mutants, and those environmentally impressed. Gale (2) and Sevag (10) have reviewed and discussed the numerous aspects of this subject.

The present communication reports studies on the influence of growth conditions on the dissimilation of the intermediary metabolites, pyruvic and oxalacetic acids, by cell suspensions of *Aerobacter indologenes*.

These studies were the outgrowth of investigations of the dissimilation of citric acid and of glucose under various environmental conditions (1, 7, 8, 9). The methods and techniques used were those employed in the papers cited.

Mickelson, Reynolds and Werkman (6) showed that *A. indologenes* dissimilated pyruvic acid anaerobically to products resembling those of the fermentation of glucose by this organism. Cell suspensions grown in glucose broth were used. Since pyruvate is in an oxidized state with respect to glucose, the products presented an analogous relationship, but a substantial yield of 2,3-butyleneglycol was obtained. Large quantities of this glycol are formed from glucose by *Aerobacter* but only very small yields of glycol are formed from citrate (1). Conversely, the fermentation of citrate produces succinic acid, whereas the normal unbuffered glucose dissimilation does not.

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\* Journal Paper No. J-1470 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 862.

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If pyruvic acid is a common intermediate in these two fermentations, as postulated, there must be significant differences in its dissimilation in the two cases. This difference might be due to the greater carbon dioxide concentration (from decarboxylation of oxalacetic acid) in the fermentation of citrate.

## RESULTS

### *Dissimilation of Pyruvate and Oxalacetate by Cells Grown in Citrate Media*

Table I presents the results of an experiment to test the foregoing hypothesis. Oxalacetate (self-buffered), pyruvate in sodium bicarbonate buffer, and pyruvate in phosphate buffer, were used as substrates with suspensions of *A. indologenes* grown in citrate medium. The data

TABLE I  
*Anaerobic Dissimilation of Oxalacetic and Pyruvic Acids by Non-proliferating Aerobacter indologenes*

Substrate dissimilated	Type of buffer	Initial CO <sub>2</sub>	mM products/100 mM substrate dissimilated							Carbon recovery	Redox index
			H <sub>2</sub>	CO <sub>2</sub>	Formic acid	Acetic acid	Lactic acid	Succinic acid	2,3-Butylene glycol		
		mM								Per cent	
7.32 mM oxalacetate	self-buffered	0	0	126.2	25.8	59.3	4.0	33.9	0.3	104.7	1.04
30.6 mM pyruvate	0.1 M phosphate	0	*	73.6	0	68.7	1.6	14.2	2.7	94.5	*
30.6 mM pyruvate	0.8% bi-carbonate	102.7	0	130.8	52.6	76.0	5.4	5.7	2.4	95.4	1.02
75.0 mM citrate <sup>a</sup>	self-buffered	0	7.0	142.8	14.8	155.0	—	21.6	5.6	97.4	0.98

Cells grown 5 days in liquid citrate medium. Duration of experiment 2.5 days.

\* Hydrogen was not determined. Gaseous products were flushed continuously from the phosphate-buffered experiment to maintain a low carbon dioxide concentration.

<sup>a</sup> Dissimilation of citric acid by cell suspension of *A. indologenes*. Taken from Brewer and Werkman (1). 1.6 mM acetylmethylcarbinol was also produced but it is not shown above.

obtained from the oxalacetate dissimilation agree with those from citrate dissimilation (1) minus the one mole of acetate split off during the conversion of citrate to oxalacetate. The yield of succinic acid from oxalacetic acid was distinctly higher than from citric acid. The effect of added carbon dioxide on pyruvate dissimilation appears to be the reverse of that expected. More succinic acid was formed in phosphate buffer than in bicarbonate. The reason is not apparent, although the latter case may have been due to a deficiency of phosphate for enzymic activity. Wood and Werkman (12) have found that phosphate stimulates the formation of succinic acid from glycerol in bicarbonate buffer by *Propionibacterium pentosaceum*.

The products from pyruvate by *A. indologenes* (Table I) are in substantial agreement with those from citrate minus the one mole each of CO<sub>2</sub> and acetic acid split off in the conversion of citric acid to pyruvic acid. The pyruvate dissimilation of Table I differs significantly from that reported by Mickelson, Reynolds and Werkman (6). These authors found that 100 mM of pyruvate were dissimilated to 56.9 mM of acetic acid, 18.6 mM of 2,3-butylene glycol, 95.9 mM of carbon dioxide and 49.1 mM of hydrogen. They did not determine succinic acid but little or none could have been present, judging from the satisfactory carbon and redox balances.

The only important differences between the conditions of the experiment of Mickelson, Reynolds and Werkman (6) and that shown in Table I was in the nature of the medium in which the cells were grown. The products in each case resembled those normally obtained in the fermentation of the growth substrate by *A. indologenes*.

#### *Dissimilation of Pyruvate, Oxalacetate, Citrate and Glucose by Cells Grown in Glucose in Acid and Alkaline Buffers and in Citrate*

Mickelson and Werkman (7) have shown that the dissimilation of glucose by *A. indologenes* differs remarkably in acid and alkaline media. They found that, under alkaline conditions, little 2,3-butylene glycol but considerable quantities of formic, acetic and succinic acids were formed. Thus, the products of the dissimilation of glucose in alkaline media resemble those of the fermentation of citric acid.

The relationships of the enzymic properties of cell suspensions of *A. indologenes* grown in different media were investigated as described below. Three types of growth substrates, citric acid, glucose in acid buffer (normal dissimilation) and glucose in alkaline buffer, were employed for the preparation of cell suspensions. Three to five days were required for the decomposition of 0.5% sodium citrate. One per cent glucose, allowed to develop acidity by normal processes, was dissimilated in 24 hours. One per cent glucose maintained at a pH above 7.5 by addition of sodium hydroxide was fermented in 14 hours. Small quantities of peptone were added to all growth media. The yield of cells from glucose media was larger than from citrate.

The activity of the cells on each of the test substrates was tested manometrically using the Barcroft-Warburg apparatus before setting up experiments for chemical analysis. Results of typical experiments are presented in Table II, along with balance sheets of fermentations by proliferating cells for comparison.

TABLE II

*Effect of Growth Substrate on the Dissimilation of Glucose and Citric, Oxalacetic and Pyruvic Acids by Cell Suspensions of Aerobacter indologenes*

Test substrate	Growth substrate	mM products/100 mM substrate dissimilated								Carbon recovery	Redox index
		H <sub>2</sub>	CO <sub>2</sub>	Formic acid	Acetic acid	Lactic acid	Succinic acid	Ethyl alcohol	2,3-Butylene glycol		
Glucose in acid buffer	Acid glucose	48.5	149.0	18.7	3.4	1.2	18.2	56.0	58.6	<i>Per cent</i> 99.5	1.00
	Alkaline glucose	22.7	67.0	20.8	30.8	97.5	0	38.6	11.3	95.1	1.15
	Citrate	45.6	61.8	0.6	38.0	82.9	15.2	36.5	11.7	94.6	0.91
Glucose in alkaline buffer	Acid glucose	39.9	31.9	100.0	77.0	1.3	39.4	61.0	5.3	98.4	1.14
	Alkaline glucose	14.0	31.3	72.8	55.2	58.6	25.2	55.5	4.1	103.4	1.15
	Citrate	41.6	43.2	67.3	65.0	43.8	22.2	56.5	1.0	96.3	1.12
Citrate	Acid glucose	6.7	143.4	16.5	166.4	2.0	24.4	0	4.1	102.1	1.03
	Alkaline glucose	6.7	132.9	27.8	168.1	1.5	24.4	0	1.9	101.1	0.94
	Citrate	6.2	127.4	24.0	147.9	0.5	22.6	0.7	3.8	92.9	0.94
Oxalacetate	Acid glucose	6.0	121.4	7.0	41.1	1.6	39.0	3.4	4.4	98.9	0.96
	Citrate	0	126.2	25.8	59.3	4.0	33.9		0.3	104.7	1.04
Pyruvate	Acid glucose	7.3	73.5	19.8	49.5	0.6	5.6	4.8	20.0	102.1	0.98
	Alkaline glucose	12.7	73.8	19.3	66.7	3.4	5.4	16.8	6.4	101.8	1.04
	Citrate	*	73.6	0	68.7	1.6	14.2		2.7	94.5	*
	Acid glucose*	41.5	164.0	16.4	0.7	4.3	0	58.2	64.5	95.5	0.98
	Alkaline glucose*	21.4	28.1	127.0	68.4	8.0	5.0	69.0	0.5	78.8	1.18
	Citrate*	7.9	132.5	33.9	162.2	1.8	17.9	1.2	2.1	96.4	1.00

\* Hydrogen was not determined.

\* Dissimilation by proliferating cells included for comparison.

It is evident that marked differences occur in the proportions of the metabolic products obtained from the dissimilation of the test substrates under conditions which are identical except for the substrate in which the cells were grown. By comparison with the typical fermentations of the growth substrates by growing cells, it appears that the nature of the dissimilation of the test substrate resembles that of the growth substrate.

The normal dissimilation of glucose by *A. indologenes* produces large quantities of 2,3-butylene glycol and ethyl alcohol with little or no acetic, lactic and succinic acids. When this dissimilation is carried out under alkaline conditions by addition of NaOH when needed, very little glycol is formed, but a large quantity of acetic acid and smaller

quantities of succinic and lactic acids are produced, accompanied by a larger yield of ethyl alcohol than in the acid medium. Very small quantities of 2,3-butylene glycol, ethyl alcohol and lactic acid are produced in the dissimilation of citrate; instead, relatively large quantities of acetic and succinic acids are formed.

The dissimilation of glucose in 0.1 *M* phosphate buffer at pH 6.3 by non-proliferating cell suspensions resulted in the formation of products which agree in general with those formed from the growth substrates. For example, 58.6 mM of 2,3-butylene glycol were formed by cells grown in glucose in acid buffer, whereas with cells grown in glucose in alkaline buffer or citrate, only 11.3 and 11.7 mM were formed. Equally striking differences were found in the yields of acetic and lactic acids with the cell suspensions grown in the different media. Cells grown in acid glucose media formed only small quantities of lactic acid from glucose in either acid or alkaline buffer, whereas cells grown in alkaline glucose or citrate produced substantial amounts of lactic acid under similar conditions.

The dissimilation of citric acid by these 3 suspensions of bacteria resulted in products which were similar in all 3 cases. Microrespirometric experiments showed that cells grown in citrate attacked citrate much more rapidly than cells grown in glucose. However, during the several days duration of these experiments, differences in rates were of little significance in the final results.

The dissimilation of oxalacetic and pyruvic acids by suspensions of *A. indologenes* grown on the various substrates is of importance because these compounds have been postulated as intermediates in the fermentation of citric acid and of glucose. One of the requisites of a true intermediate is that it can be dissimilated to the same products as the parent substrate (except that oxidation-reduction relationships may modify the quantitative relationships of the products). Since the dissimilations of glucose and of citric acid by proliferating *A. indologenes* are not identical, the intermediately formed pyruvate must be converted to different final products in each of the cases. The data of Table II indicate that this assumption is true.

Larger quantities of 2,3-butylene glycol were formed from pyruvate by cells grown in glucose in acid buffer than by cells grown in glucose in alkaline buffer or in citrate, a fact which agrees with the fermentations of these compounds by proliferating cells. Cells grown in citrate produced more succinic acid from pyruvate than glucose-grown cells did, again in conformity with the fermentations by growing cells.



Oxalacetic acid has not been postulated as an intermediate in the anaerobic dissimilation of glucose by *A. indologenes* but the dissimilation of oxalacetate by cells grown in glucose is of interest as a comparison with that of cells grown in citrate. Since this compound is both an  $\alpha$ - and a  $\beta$ -keto acid, it is subject to spontaneous  $\beta$ -decarboxylation. Thus, a portion of the  $\text{CO}_2$  liberated may not be the result of enzymic activity. The products of the dissimilation of oxalacetate by the two types of *A. indologenes* cell suspensions used were similar. Rather high yields of succinic acid were obtained in both cases. As in the dissimilation of pyruvate by the different types of cells, the yield of acetic acid was smaller and the yield of 2,3-butylene glycol was larger with cells grown in glucose as compared with those grown in citrate. Such agreement is to be expected, since pyruvate is closely related chemically to oxalacetate and is formed non-enzymically by the decarboxylation of the latter.

In general, each enzymic variant acted most vigorously on the same substrate in which it was grown. For instance, alkaline glucose cells fermented only 11 of 30 mM of glucose (0.1 M) in acid buffer in one week. The same cells completely fermented 0.1 M glucose in alkaline buffer in less than 2 hours.

In general, citrate and alkaline glucose cells behaved similarly on all the substrates investigated and both were unlike acid-grown glucose cells in their metabolism. It appears that *A. indologenes* develops predominantly a glycol-forming mechanism when grown in glucose under acid conditions. In citrate and under alkaline conditions in glucose, an acid-forming mechanism is similarly developed and the ability to form glycol is not acquired to any appreciable degree.

#### *Effect of Added Acetaldehyde on the Dissimilation of Oxalacetic Acid*

The differences between the fermentations of glucose in acid buffer and citrate may in part be due to the relative states of oxidation and reduction of the two substrates. It is possible that the differences observed may result from a relative shortage of hydrogen donors in the citrate fermentation. This point was investigated by the addition of acetaldehyde to a dissimilation of oxalacetic acid by non-proliferating *A. indologenes*. The results (Table III) show that the addition of acetaldehyde did not appreciably affect the distribution of carbon among the products except in the case of ethyl alcohol and acetic acid.

TABLE III

*Effect of Added Acetaldehyde on the Anaerobic Dissimilation of Oxalacetic Acid by Non-proliferating Aerobacter indologenes*

Oxalacetate dissimilated	Acetaldehyde added		Aldehyde recovered	mM products/100 mM of substrate dissimilated								Carbon recovery	Redox index
	to fermentation	per 100		H <sub>2</sub>	CO <sub>2</sub>	Formic acid	Acetic acid	Ethyl alcohol	2,3-Butylene glycol	Lactic acid	Succinic acid		
mM 15.0 10.5	mM 0 13.3	mM 125.7	mM 0	6.0 4.1	121.4 145.8	7.0 0	41.1 91.8	3.4 72.7	4.4 2.3	1.6 2.3	39.0 43.2	Per cent 98.9 101.9	0.96 1.01

Cells grown in glucose medium. Duration of experiment 7 days.

The yields of both of these compounds were greatly increased by the addition of aldehyde. It is evident that the acetaldehyde was subjected to dismutation. The excess of alcohol was balanced by an increase in the oxidized compounds, succinic acid and carbon dioxide. Hammer (3) showed that when aldehydes were added to milk cultures of *Streptococcus liquefaciens*, higher yields of acetylmethylcarbinol were found. Acetaldehyde added to glucose fermentations by *A. indologenes* caused an increase in the yield of acetylmethylcarbinol (8). Mickelson (5) later showed that the addition of acetaldehyde to pyruvate dissimilations by non-proliferating *A. indologenes* did not affect the small yield of glycol. The data of Table III show likewise that added aldehyde has no significant effect on the formation of 2,3-butylene glycol.

## DISCUSSION

From these experiments, it appears that the enzymic constitution of cultures of *A. indologenes* is influenced by the conditions under which the organisms are grown. Growth on one substrate affects the activity of the organism on other substrates. This principle is an extension of Karström's theory of adaptive and constitutive enzymes. The enzymic activity of an organism on a specific substrate is affected not alone by growth on the same substrate but also by growth on other compounds.

From the evidence presented it is not possible to determine the exact nature of the enzymic variation demonstrated. Possibly the supposedly pure culture of *A. indologenes* carried a constant, small proportion of variant cells which became predominant under favorable

conditions of growth. A more probable explanation is that the bacterial cells were altered in their enzymic constitution by growth on certain substrates. Some indication that adaptation of "non-proliferating" cells occurred is given by the experiments with citrate. Cells grown in glucose media were practically inactive on citrate in microrespirometric experiments of short duration, yet the same cells completely dissimilated 0.1 *M* citrate over a period of 6 days.

In the dissimilation of glucose, the glycol-forming enzyme system is relatively active but the enzyme system for the formation of succinic acid is relatively inactive; in the citrate fermentation the relative concentrations of 2,3-butylene glycol and succinate are reversed. The fact that pyruvate is dissimilated, under conditions which are experimentally identical except for the nature of the growth substrate, to unlike final products indicates that the two types of cells are enzymically different.

#### SUMMARY AND CONCLUSIONS

1. The nature of the enzymic activity of non-proliferating cell suspensions of *Aerobacter indologenes* on glucose in alkaline and in acid buffer, on citrate, oxalacetate and pyruvate is dependent on the nature of the substrate on which the cells are grown.

2. Cell suspensions grown in glucose in acid and in alkaline buffers, and in citrate, dissimilate other substrates to products resembling those formed from the growth substrate.

3. Pyruvate is dissimilated by *A. indologenes* cells grown in citrate, glucose in acid buffer, and glucose in alkaline buffer to products which, in each case, resemble the usual fermentation of the growth substrate by proliferating cells.

4. These facts may explain the formation by one bacterial strain of diverse products from several substrates through mechanisms which employ in part the same enzymic reactions.

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# **The Determination of Plasma and Serum Protein by Electrophoresis. The Effect of Protein Concentration and Voltage Changes on Proportions of Different Proteins\***

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Received April 1, 1947

## **INTRODUCTION**

The purpose of this electrophoretic study was to modify Tiselius' (1) method so as to determine the proportions of proteins as they exist in the circulating blood by eliminating any effects which might be artifacts due to separation of fibrin, dilution, high voltage, or buffer.

Tiselius found in 1938 that an albumin, very small globulin fractions and a large immobile fraction separated from the boundary between undiluted horse serum and the same serum slightly diluted. The results were not given quantitatively. He found that under these conditions boundary anomalies were not important.

Following a method described under *Experimental* and suggested by Tiselius' experiments, we studied the effects of dilutions on the electrophoretic pattern of serum. As the serum dilutions were made progressively greater, the changes were chiefly an increase in albumin and a simultaneous decrease in  $\gamma$ -globulin.

We also studied the effects of changes in voltage.

## **EXPERIMENTAL**

Chicken plasma and serum, rat serum and human serum were used in the experiments, which were run immediately after collection of the blood plasma or serum, before any changes were likely to have taken place.

We studied the effect of dilution on the electrophoretic patterns of serum, using an

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apparatus made up with two middle cells. Dilutions of serum with buffer in the following proportions were made:

Ml. of Serum	Ml. of Buffer
5.....	0
4.....	1
3.....	2
2.....	3
1.....	4
0.....	5

Boundaries were established between different dilutions of serum, instead of the usual boundaries between serum and buffer. The bottom cell and one side of the middle cell, just above the bottom cell, were filled with undiluted serum. On both sides the compartments just above the undiluted serum were filled with the dilution made with 4 ml. serum to 1 ml. buffer. The remaining side of the top middle cell was filled with buffer. Ringer's solution, adjusted to pH 7.4, was used as a diluent and as the buffer in order to have all conditions as nearly as possible like those in the blood. When the cells were moved into alignment, boundaries between undiluted serum and diluted serum were established. After electrophoresis the pictures were taken of the lower middle cell. In this same manner each dilution of serum was studied with the one next to it in the series. Each boundary represented a difference in protein concentration of about 1.3 g.-% of protein.

### COMPARISON OF CHICKEN PLASMA AND SERUM

Since non-clotting vertebrate blood plasma can be obtained without the use of an anticoagulant only from birds, and then only with parafined canula and tubes, Dr. T. Addis suggested that chicken plasma and serum be compared to see whether there was any change in the proportions of the proteins in the electrophoretic patterns due only to the separation of the fibrin. The chicken plasma was taken from the femoral artery without the addition of an anticoagulant. The only significant difference between the patterns of chicken plasma and serum appeared to be the elimination of the fibrinogen peak of the chicken plasma and a simultaneous increase in the  $\gamma$ -globulin (Table I, Fig. 1). The simplest explanation of this effect of the increase in  $\gamma$ -globulin at the expense of fibrinogen would be that part of the  $\gamma$ -globulin is attached to the fibrinogen in the plasma.

Therefore, it appeared more reasonable to substitute for the unobtainable, non-coagulating plasma of other vertebrates the easily obtainable serum than to use an anticoagulant which might change the pattern.

Koenig and Hogness (2) identified as  $\beta_2$ -globulin in veronal buffer the part of the fibrinogen in swine plasma which is not removed on clotting. In phosphate buffer it is apparently included in the  $\gamma$ -globulin. Hogness, Griffie and Koenig (3) found the part of the fibrinogen remaining after removal of the fibrin from bovine plasma by

TABLE I

*Comparison of Electrophoretic Patterns of Chicken Plasma and Serum  
Showing Increase of  $\gamma$ -Globulin After Separation of Fibrin*

Plasma				Serum			
Dilution	Fractions	Per Cent	Plasma Protein by Nessler's Method—Times Per Cent	Dilution	Fractions	Per Cent	Plasma Protein by Nessler's Method—Times Per Cent
<i>Experiment No. 507</i>				<i>Experiment No. 508</i>			
undiluted	albumin	38.3	.473	undiluted	albumin	38.9	.375
1-1	$\alpha$	16.0	.198	1-1	$\alpha$	18.9	.185
	$\beta$	5.02	.062		$\beta$	8.03	.08
	fibrinogen	22.9	.284		$\gamma$	34.2	.33
	$\gamma$	17.9	.220				
<i>Experiment No. 515</i>				<i>Experiment No. 516</i>			
1-1	albumin	28.0	.334	1-1	albumin	27.4	.308
buffer	$\alpha$	16.1	.192	buffer	$\alpha$	16.5	.186
	$\beta$	7.55	.090		$\beta$	10.4	.117
	fibrinogen	27.4	.327		$\gamma$	45.7	.514
	$\gamma$	20.9	.249				
<i>Experiment No. 520</i>				<i>Experiment No. 521</i>			
1-1	albumin	24.3	.38	1-1	albumin	31.1	.45
undiluted	$\alpha$	10.7	.17	undiluted	$\alpha$	8.91	.13
	$\beta$	7.95	.13		$\beta$	10.4	.15
	fibrinogen	27.8	.44		$\gamma$	49.5	.72
	$\gamma$	29.3	.46				
<i>Average</i>				<i>Average</i>			
	albumin	30.2	3.94		albumin	32.4	.378
	$\alpha$	14.3	.187		$\alpha$	14.7	.167
	$\beta$	6.84	.094		$\beta$	12.9	.116
	fibrinogen	26.0	.350		$\gamma$	43.1	.521
	$\gamma$	22.7	.310				



continued stirring to be a separate fraction,  $\gamma$ -globulin. The mobility of the  $\beta_2$ -globulin in the first case and of the  $\gamma_1$ -globulin in the second case are nearly that of the respective fibrinogens.

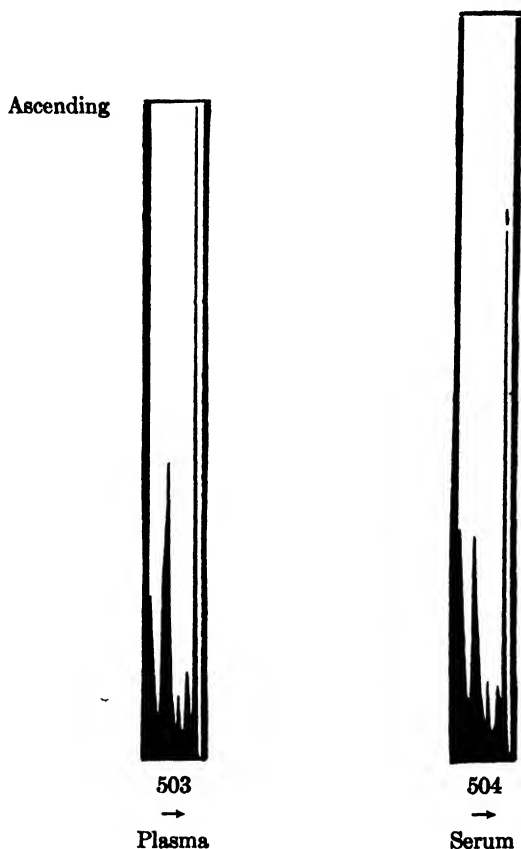


FIG. 1. Electrophoretic patterns of chicken plasma and chicken serum. The albumin is at the right of the picture. Buffer: Ringer's Solution, pH 7.4. Current: 30 m.a.

### DILUTION

Table II shows the effect of dilution on the proportions of the proteins in chicken plasma and serum without the immobile fraction. There is a decrease in the  $\gamma$ -globulin alone and a slight increase in albumin,  $\alpha$ -globulin and  $\beta$ -globulin. The change in the serum on dilution does not differ appreciably from the change in plasma.

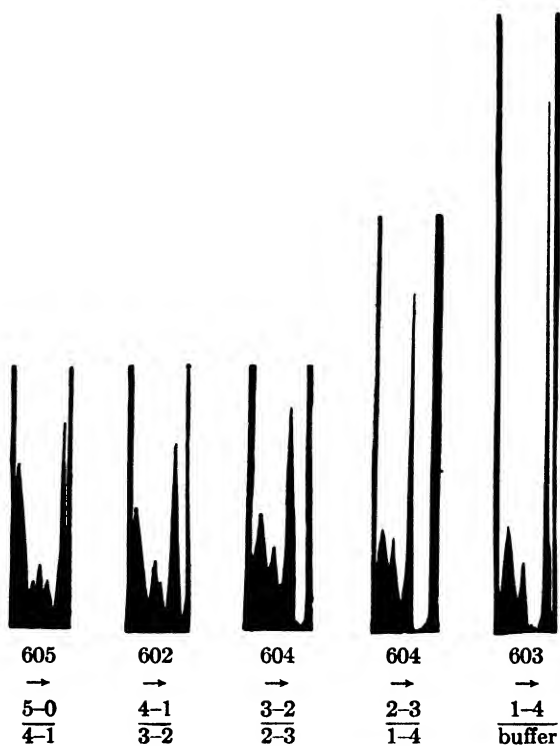
TABLE II  
*Effect of Dilution on Proportions of Electrophoretic Fractions of  
 Chicken Plasma and Serum*

Plasma			Serum		
Dilution			Dilution		
$\frac{1-1}{\text{undiluted}}$		$\frac{\text{buffer}}{1-1}$	$\frac{1-1}{\text{undiluted}}$		$\frac{\text{buffer}}{1-1}$
<i>Experiment No. 507</i>			<i>Experiment No. 508</i>		
albumin	per cent 37.0	per cent 43.4	albumin	per cent 34.8	per cent 35.5
$\alpha$	16.9	24.2	$\alpha$	18.2	22.5
$\beta$	4.48	2.93	$\beta$	7.03	8.86
$\gamma$ +fibrinogen	41.5	29.5	$\gamma$	40.0	33.2
<i>Experiment No. 515</i>			<i>Experiment No. 516</i>		
albumin	31.1	33.9	albumin	31.4	34.3
$\alpha$	16.5	19.3	$\alpha$	14.4	17.9
$\beta$	7.77	11.4	$\beta$	7.21	11.5
$\gamma$ +fibrinogen	44.6	35.4	$\gamma$	46.9	36.3
<i>Experiment No. 520</i>			<i>Experiment No. 521</i>		
albumin	24.3	26.2	albumin	31.1	31.6
$\alpha$	10.7	11.7	$\alpha$	8.91	15.7
$\beta$	7.95	9.76	$\beta$	10.4	10.5
$\gamma$ +fibrinogen	57.0	52.3	$\gamma$	49.5	42.1
<i>Average</i>			<i>Average</i>		
albumin	30.8	34.5	albumin	32.4	33.8
$\alpha$	14.7	18.4	$\alpha$	13.8	18.7
$\beta$	6.77	8.68	$\beta$	8.21	10.3
$\gamma$ +fibrinogen	47.7	39.1	$\gamma$	45.5	37.2

Table III and Figs. 2 and 3 give the results of dilution of rat and human serum. The albumin and  $\alpha$ -globulin<sup>1</sup> and  $\beta$ -globulin increase at the expense of the  $\gamma$ -globulin. It may be suggested that when we are considering only the percentages of the proteins, a decrease in  $\delta$ -boundary or  $\epsilon$ -boundary, which are not always separated from the  $\gamma$ -globulin, might give an apparent increase in the percentages of the

<sup>1</sup> Rat  $\alpha$ -globulin is questionable as it is not well separated.

# Ascending



# Descending

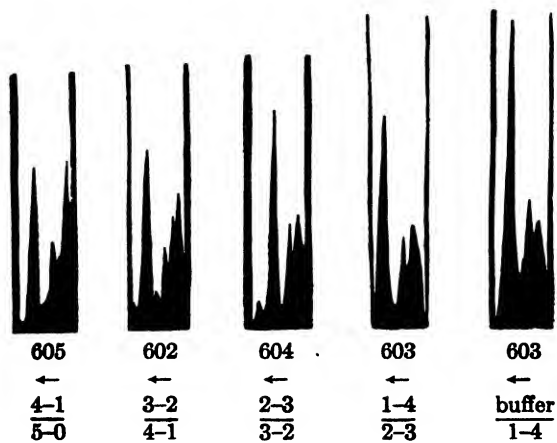
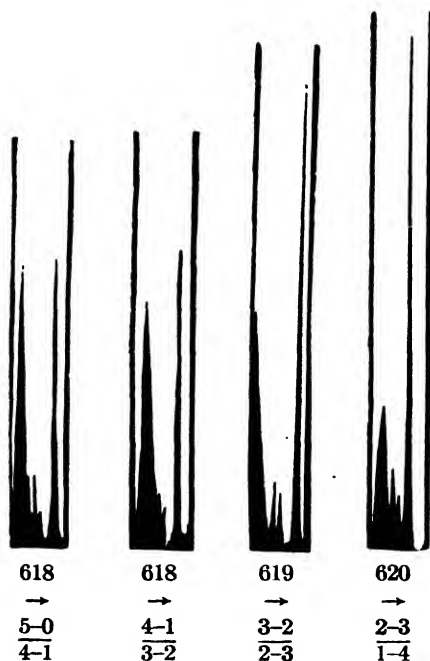


FIG. 2. Electrophoretic patterns of rat serum at different dilutions. On the ascending side the albumin is at the right of the pictures. On the descending side the albumin is at the left of the pictures. Buffer: Ringer's Solution, pH 7.4. Current: 30 m.a.

Ascending



Descending

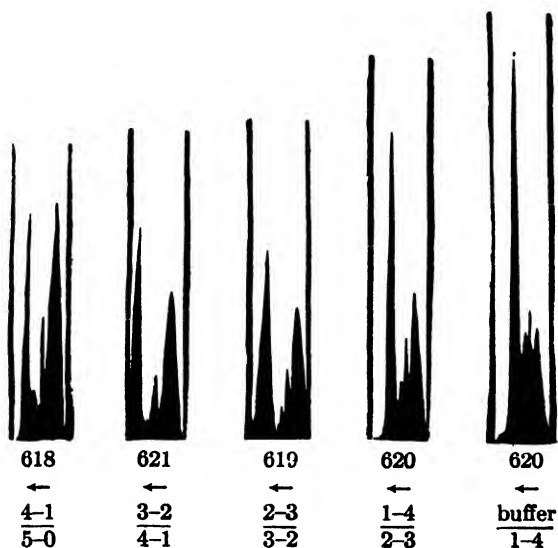


FIG. 3. Electrophoretic patterns of human serum at different dilutions. On the ascending side the albumin is at the right of the pictures. On the descending side the albumin is at the left of the pictures. Buffer: Ringer's Solution, pH 7.4. Current: 30 m.a.

other fractions. However, there is the same proportional increase in the areas under the albumin peak. These are, of course, independent of the  $\delta$ -boundary and the  $\epsilon$ -boundary.<sup>2</sup>

TABLE III  
*Comparison of Percentages of Electrophoretic Fractions of  
Serum at Different Dilutions*

<sup>1</sup> Dilutions (Average of positive and negative sides)

Buffer pH	m.a.	$\frac{5-4}{4-1}$ and $\frac{4-1}{3-0}$	$\frac{4-1}{3-2}$ and $\frac{3-2}{4-1}$	$\frac{3-2}{2-3}$ and $\frac{2-3}{3-2}$	$\frac{1-4}{2-3}$ and $\frac{2-3}{1-4}$	$\frac{1-4}{\text{buffer}}$ and $\frac{\text{buffer}}{1-4}$
Human Serum						
Ringer's 7.4	30	Experiment No. 618	Experiment No. 612	Experiment No. 619	Experiment No. 620	Experiment No. 620
		<i>per cent</i> albumin 35.6	<i>per cent</i> 40.9	<i>per cent</i> 47.3	<i>per cent</i> 49.4	<i>per cent</i> 50.2
		$\alpha$ 4.41	3.21	4.83	5.82	8.04
		$\beta$ 7.24	7.43	7.94	10.1	14.2
		$\gamma$ 52.5	48.4	40.0	34.6	27.5
Rat Serum						
Ringer's 7.4	30	Experiment No. 605	Experiment No. 602	Experiment No. 604	Experiment No. 603	Experiment No. 603
		<i>per cent</i> albumin 35.9	<i>per cent</i> 36.6	<i>per cent</i> 46.0	<i>per cent</i> 50.0	<i>per cent</i> 54.6
		$\alpha$ 7.20	13.5	—	—	4.06
		$\beta$ 10.6	11.0	13.9	14.6	13.4
		$\gamma$ 46.4	45.7	40.2	34.2	28.0

The increase in albumin in chicken plasma and serum is less than that in either rat or human serum. The fact that undiluted chicken plasma and serum have a low protein content, so that the dilution is only from about 3.0–1.5 mg.-%, instead of from 6.4–1.28 mg.-% as in rat serum, would make the total increase much smaller.

<sup>2</sup> The areas of each graph should be approximately equal, as the difference in concentration of protein between the two adjacent dilutions of serum are very nearly the same.

## EFFECT OF LOWERING THE VOLTAGE

To still further maintain unchanged physiological conditions, to observe the effect of voltage on rat and human serum, and, therefore, test the stability of serum proteins, the current was lowered from 30 to 2 milliamperes. (See Table IV and Fig. 4.) The percentage of albumin

TABLE IV  
*Comparison of Percentages of Electrophoretic Fractions of Serum on Dilution at 2 m.a.*

Dilutions (Average of positive and negative sides)

Buffer pH	m.a.	5-0 and 4-1 4-1 and 5-0	---	3-1 and 2-2 2-2 and 3-1	2-3 and 1-3 1-3 and 2-3	1-2 buffer and 1-2 buffer and 1-2
Ringer's 7.4	2	Experiment No. 623 (Rat serum) per cent albumin 31.7		Experiment No. 636 (Human serum) per cent albumin 47.6		
			---	per cent albumin 56.2	per cent albumin 60.9	

in the undiluted rat serum was not greatly changed by lowering the voltage. A comparison of similar dilutions of human serum also showed increases in the percentages of albumin with increasing dilution. There was an incomplete separation of globulins, but that may have been due partly to the diffusion taking place during the greatly increased length of the time of the experiments. The  $\alpha$ -globulin was not visible in the human serum. The turbidity of the rat serum, which increased during the experiment, made the proportions of the globulins questionable, if, indeed, they were separated at all. The effect of voltage will be studied further.

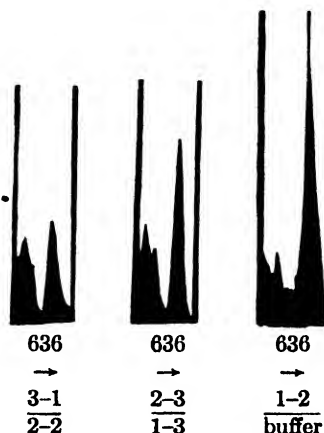
## DISCUSSION

Perlmann and Kaufman (4), in their study of the effect of protein concentration in barbiturate buffer, found that the percentages of albumin increased slightly with the concentration. In our experiments we find this also true in Ringer's solution when the boundary is between protein solutions and buffer as in his studies. We have data only on the descending side. The change is from 66% with a dilution of 4 ml. of serum to 1 of buffer; 64% with a dilution of 3 ml. of serum to 2 of buffer; 61% with a dilution of 1 ml. of serum to 4 of buffer. This effect is very small and in the opposite direction to that found when the

boundaries are between two protein concentrations as in our tables and figures.

As suggested in 1942 (5), there are two distinct parts to the  $\gamma$ -globulin, one of which (that of slower mobility) has a solubilizing

Ascending



Descending

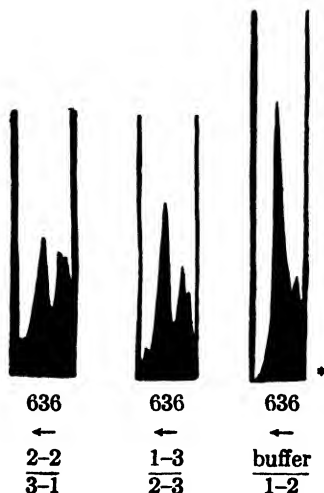


FIG. 4. Electrophoretic patterns of human serum at different dilutions. On the ascending side the albumin is at the right of the pictures. On the descending side the albumin is at the left of the pictures. Buffer: Ringer's Solution, pH 7.4. Current: 2 m.a.

\* Gamma globulin is outside of picture.

action on the other part. Dilution probably lessens this action and makes the colloidal system more unstable, allowing the combination of euglobulin and albumin to take place. McFarlane (6) found the effect of dilution reversible in his experiments on sedimentation.

As further evidence of a combination of globulin and albumin the experiments of Grönwall (7) are offered. He separated euglobulin from albumin and pseudoglobulin. He showed that the solubility of euglobulin in isotonic NaCl is increased by the presence of albumin. The splitting of the protective pseudoglobulin from euglobulin was irreversible, as there was no increase of solubility of euglobulin when pseudoglobulin was added to the isotonic salt solution.

In discussing the relationship of  $\gamma$ -globulin to albumin, reference should be made to the role of albumin in immune reactions and especially with regard to the reaction of albumin and complement. Davis (8) states: "Neither antigen nor antibody alone fixes complement. But it was found that, whereas the antibody added in the form of serum does not by itself interfere with complement, the addition to complement of electrophoretically separated  $\gamma$ -globulin (the fraction of serum which contains most of the antibody) is 'anti-complementary' even in high dilution—i.e., even in the absence of antigen it prevents the subsequent reaction of the complement with sensitized red cells. Addition of albumin to the  $\gamma$ -globulin, however, in approximately the proportion initially present in the serum, restores its inert behavior toward complement in the absence of antigen." This action of albumin on  $\gamma$ -globulin is a protective one.

McFarlane (6), in discussing the effect of dilution on the sedimentation of the albumin and globulin mixtures in the ultracentrifuge, said: "It is believed that there exists in a mixture of albumin and globulin a form of molecular dimensional equilibrium, the exact nature of which still has to be discovered." In his experiments on diluting serum (human, cow or horse) with 1% of NaCl the percentage of globulin increased at the expense of the albumin and X (the X fraction sediments a very little faster than albumin and is not always separated from it). The greatest increase was in horse serum. All these experiments have shown that part of the globulin (probably euglobulin) can be associated with either the  $\gamma$ -globulin or albumin.

Our experiments throw further light on this equilibrium. We found that, in the electrophoretic pattern, the albumin fraction increased on dilution at the expense of the  $\gamma$ -globulin. We suggest, as a first approximation to a picture of what is undoubtedly a very complex relationship, that part of McFarlane's albumin or X (probably X) has the mobility



of  $\gamma$ -globulin with a molecular size only slightly greater than albumin, so that it sediments with the albumin, but travels in the electric field as part of the  $\gamma$ -globulin. On dilution, a combination of this  $\gamma$ -globulin or X with albumin makes a particle of molecular size approaching that of globulin and consequently sediments with the globulin, at the same time assuming a charge very nearly like that of the albumin (as quartz particles do when coated with egg albumin) (9). Therefore, it travels in the electrophoretic experiments with the albumin. This is in agreement with the many observations that colloidal osmotic pressure becomes less on dilution than is accounted for by the dilution itself. It might be that the attachment of part of the  $\gamma$ -globulin to the fibrinogen helps to keep the fibrinogen in solution in the plasma.

The work of Jayle and Judas (10) suggests that the protein transferred from globulin to albumin may be a glycoprotein.

Koenig, Perrings and Hogness (11) investigated the fractions separated by electrophoresis from bovine plasma using different buffers. They found an interaction of the proteins with themselves and an interaction of the proteins with the buffer. When artificial mixtures of albumin,  $\gamma$ -globulin and fibrinogen were analyzed in the same manner, the interaction was very much less. It is difficult to compare their results with ours as the interactions appear to be different. The principal interaction in their experiments is between albumin and the  $\alpha$ -globulins, while in ours it is between the albumin and  $\gamma$ -globulin.

In order to make an exhaustive study of a serum or plasma it appears necessary to study it in different buffers and at different protein concentrations. Only then can the presence of normal or abnormal interactions between the proteins be determined.

### SUMMARY AND CONCLUSIONS

1. The only significant change in the pattern of chicken plasma brought about by the separation of fibrin appeared to be the loss of the fibrinogen peak and a simultaneous increase in the  $\gamma$ -globulin.

2. Dilution increases both the proportion and absolute quantity of albumin in human and rat serum as shown by the electrophoretic pattern. This is accompanied by a decrease in  $\gamma$ -globulin and an increase in the other globulins.

3. Chicken plasma and serum react similarly to dilution.

4. Lowering the voltage to a small fraction of that ordinarily used did not greatly change the proportion of albumin in undiluted serum.

5. The conclusion may be drawn that the customary voltage may be used with Ringer's solution, pH 7.4, as a buffer in electrophoretic experiments on undiluted serum without fear of greatly changing the proportion of albumin which occurs in the circulating plasma. However, normal or pathological sera should be studied at several dilutions and with different buffers to find out whether the normal shifts from one fraction to another take place.

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# Crystalline Lipoxidase II. Lipoxidase Activity

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Received July 21, 1947

## INTRODUCTION

Soy bean lipoxidase has recently been isolated in pure crystalline form by Theorell, Holman, and Åkeson (1, 2). The present report is concerned with the study of the mode of action of the enzyme and the influence of experimental conditions upon its action. All reactions were carried out with pure crystalline enzyme and with homogeneous sodium linoleate substrate at pH 9 unless otherwise stated. Measurement of lipoxidase activity was made using the spectrophotometric assay (3) whenever possible.

## THE INFLUENCE OF pH UPON LIPOXIDASE ACTIVITY

Sodium linoleate substrate was prepared and divided into portions which were adjusted to various pH values by addition of NaOH or HCl. The determination of activity was made in the conventional way except that the thiocyanate determination of peroxide was used because of the high ultraviolet absorption of alkaline blanks. The relative activities of the enzyme at various pH values are shown in Table I.

TABLE I

*The Effect of pH on Lipoxidase Activity*

pH	Relative activity
6.70	0.45
7.50	1.65
8.45	1.79
8.90	1.75
9.40	2.24
10.30	2.15
11.70	2.15

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It will be seen that lipoxidase is able to tolerate rather high alkalinity without loss of activity, but that the activity decreases markedly as neutrality is approached. It is likely that this effect is due to the relative inaccessibility of substrate in the emulsion systems existing below pH 9, rather than inactivation of the enzyme, because the enzyme withstood even greater acidity for long periods of time during its preparation.

#### THE INFLUENCE OF TEMPERATURE ON LIPOXIDASE ACTIVITY

To determine the effect of temperature upon the rate of reaction, a series of assays were made at each of several temperatures. The relative activities, expressed as  $D_{234}$  developed in two minutes reaction

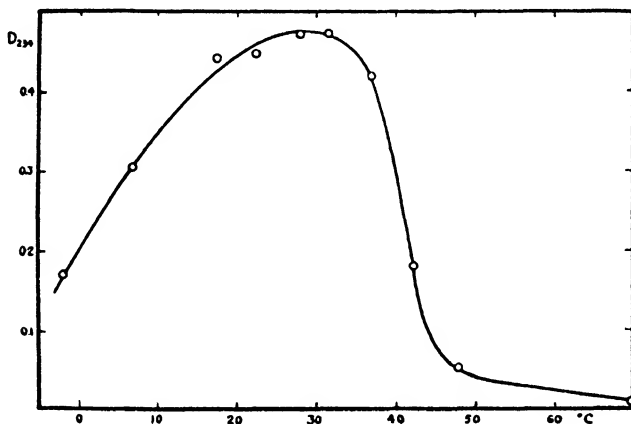


FIG. 1. The effect of temperature upon lipoxidase activity.

time, are shown in Fig. 1. It should be pointed out that the precipitous decrease in reaction rate above  $30^{\circ}\text{C}$ . does not represent a thermal destruction of the enzyme, for the enzyme endured a much higher temperature for a longer period of time in its preparation without significant loss in activity. The  $Q_{10}$  between  $-1.5^{\circ}\text{C}$ . and  $18.5^{\circ}\text{C}$ . is approximately 1.6.

In measurements of oxygen consumption at various temperatures, it was found that the initial rates of reaction were  $37^{\circ} > 20^{\circ} > 8^{\circ} > 0^{\circ}$ , but that after a few minutes the rate of reaction decreased at the higher temperatures, whereas the rates in the cold remained constant much

longer. Fig. 2 illustrates this effect, showing the oxygen uptakes at 0°C. and 37°C. using equal quantities of enzyme. The decrease in activity at the higher temperatures is apparently due to an inactivation of the enzyme in contact with substrate or reaction products. The effect of temperature on the apparent extinction coefficient of the peroxidic products will be presented below.

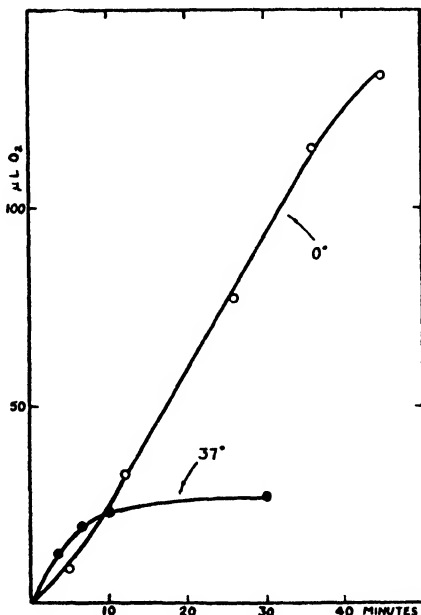


Fig. 2. Oxygen absorption of lipoxidase-linoleate system at 0°C. and 37°C.

### ENZYME INHIBITORS

Numerous studies have been conducted on the inhibition of lipoxidase, but the results obtained on emulsion systems and crude enzyme have often conflicted (4, 5, 6, 7, 8). A repetition of some of this work has been made, using a variety of substances. The activities were determined in the usual way, but using 0.5 ml. substrate containing 4.2 mg. linoleic acid/ml. and 0.5 ml. of the appropriate inhibitor solution. Use of the thiocyanate determination of peroxide was necessary in cases where ultra-violet absorption of the inhibitor was too high in the blanks.

Concentration-inactivation curves for 5 common enzyme inhibitors are shown in Fig. 3. It is apparent that none of these substances is

effective as an inhibitor of lipoxidase. The oxygen uptake of the lipoxidase-linoleate system was unaffected by the presence of 0.07 *M* diethyldithiocarbamic acid in the reaction mixture. These results, and the absence of heavy metals in lipoxidase, exclude the possibility that lipoxidase is active through a heavy metal catalysis.

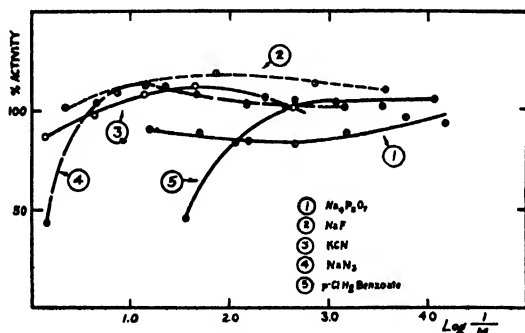


FIG. 3. Concentration-activity curves for enzyme inhibitors.

Mercury ions do not inhibit lipoxidase except in concentrations where the enzyme is precipitated, and *p*-chloromercuribenzoate inhibits only in *M*/100 concentration. The nitroprusside test indicated less sulfhydryl than 0.6% measured as free cysteine. It is thus possible to rule out sulfhydryl groups as active centers.

$\alpha$ -Naphthol gives 29% inhibition at  $2 \times 10^{-4}$  *M* and 61% inhibition at  $10^{-3}$  *M*.  $\alpha$ -Tocopherol caused a 25% inhibition at  $4 \times 10^{-4}$  *M*. It is likely, however, that this inhibition is due, not to a true enzyme inhibition, but to antioxidant action of these phenols.

#### SUBSTRATE COMPETITION

The competition between linoleic acid and related acids for the active centers in lipoxidase gives some information concerning the substrate structure to which the enzyme is attracted. A series of 4 fatty acids, which are not substrates for lipoxidase (9), and which have low melting points and consequently relatively high solubility as salts, were tested for their inhibition of the oxidation of linoleic acid under assay conditions. From the results, shown in Fig. 4, it will be seen that elaidolinolenic acid, the *trans* isomer of linolenic acid, has nearly as strong an affinity for lipoxidase as does linoleic acid. Conjugated 10,12-

linoleic acid (m.p.  $8^{\circ}\text{C}.$ ) has somewhat less affinity for the enzyme, and oleic acid has about 0.1 the affinity for lipoxidase that linoleic acid has. Octanoic acid apparently has about 0.01 the affinity for the enzyme that linoleic acid has. From these data it is possible to conclude that the enzyme has an affinity for the unsaturated centers in its substrates, and that it also has a slight affinity for either the paraffin skeleton or the carboxyl group of fatty acids. It is thus conceivable that lipoxidase activity is reduced by oleic acid and saturated acids *in vivo* when the substrates have been reduced to low concentration.

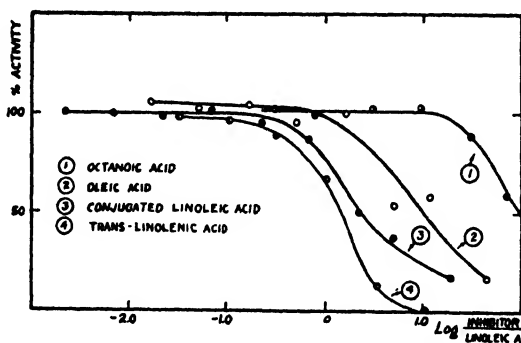


FIG. 4. Lipoxidase inhibition by substrate competition.

### LIPOXIDASE ACTIVATOR ENZYME

Lipoxidase has been shown by Balls, Axelrod, and Kies (10) and by Theorell, Bergström, and Åkeson (11) to require an activator enzyme for its maximum activity in the reaction systems used in their experiments. Kies (12) has isolated a polypeptide activator from soy beans which, under favorable conditions, accelerates the reaction 300%. These workers have used emulsion systems near neutrality, but no activator effect is demonstrable in the homogeneous system now used in this laboratory.

In the system originally used by Theorell, Bergström, and Åkeson (11), the oxygen uptake rapidly reached a plateau at which the addition of an equal quantity of enzyme increased the oxygen uptake several times that of the first addition of enzyme. However, using crystalline lipoxidase and homogeneous substrate at pH 9 and at  $37^{\circ}\text{C}.$ , this effect did not appear. The oxygen uptake upon the second addition of enzyme was merely the same as upon the first addition.

The addition of boiled extract of soy bean to homogeneous substrate, prior to the addition of pure enzyme, slightly diminished, rather than increased, the rate of oxidation. It seems likely that the activator effect is due to some surface tension phenomenon in emulsion systems, but that this activator effect is not necessary in homogeneous systems.



## SUBSTRATE SATURATION OF LIPOXIDASE

To determine the effect of linoleic acid concentration upon the rate of its oxidation with lipoxidase, potassium linoleate was used as substrate because it is more soluble than sodium linoleate. The other experimental conditions were the same as assay conditions. In Fig. 5 the reciprocal of the reaction rate is plotted against the reciprocal of linoleate concentration. It will be seen that, at high substrate concentration, the enzyme is somewhat inhibited. The substrate concentration at half-maximum rate was found to be  $K_m = 1.35 \times 10^{-3} M$ . The substrate concentration used in the assay (shown by the arrow in Fig. 5.) lies in the area where slight variations in substrate concentration have little effect upon the apparent rate of the reaction.

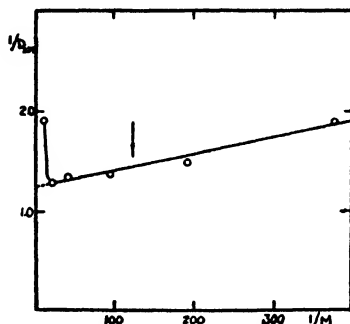


FIG. 5. Saturation of lipoxidase with linoleic acid.

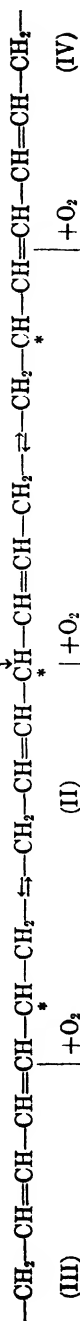
## OXYGEN SATURATION OF LIPOXIDASE

Preliminary experiments indicate that at  $0^{\circ}C.$  and in  $7 \times 10^{-3} M$  linoleate the enzyme is saturated with oxygen at about 160 mm.  $O_2$  or  $4.2 \times 10^{-4} M O_2$ . The Michaelis constant was calculated to be  $K_m = 1.2 \times 10^{-4} M$  or 40 mm.  $O_2$ . It was also found that at 30 mm.  $O_2$  the rate of reaction is proportional to enzyme concentration.

EXTINCTION COEFFICIENT OF CONJUGATED  
HYDROPEROXIDIC PRODUCTS

The development of conjugated unsaturated systems during the oxidation of linoleic acid and related substances has been demonstrated by Farmer (13), Bolland and Koch (14), Holman *et al.* (15, 16), and by Bergström (17). However, the apparent degree of conjugation found by various workers was not the same, Bolland and Koch reporting  $\epsilon =$



$$-H^*$$


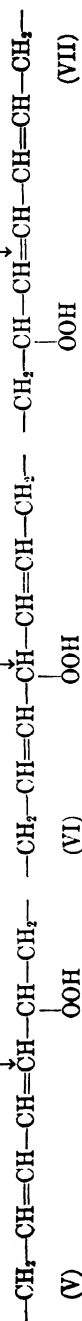
$$+O_2$$

$$+O_2$$

$$+O_2$$

$$+H^*$$

$$+H^*$$

$$+H^*$$


$$OOH$$

$$OOH$$

$$OOH$$

22,700, Bergström reporting 14,000, and Holman finding values around 19,000.

In the mechanism of oxidation of linoleic acid proposed by Bolland and Koch (14), the primary free radical can exist in 3 equilibrium forms which add oxygen to give the 3 isomeric hydroperoxides, two of which are conjugated. The value of 22,700 for the molecular extinction coefficient of the products agreed well with this hypothesis, for it is about  $\frac{2}{3}$  of the extinction coefficient reported for conjugated dienoic acids. The peroxide content of oxidized linoleates approximates the oxygen uptake, and the non-conjugated peroxide has been attributed to the direct addition of oxygen to the primary radical (II), to yield the 11-hydroperoxide (VI). In view of the quite variable degree of conjugation induced under various conditions, it was thought pertinent to determine the degree of conjugation of linoleic acid under the more ideal conditions using pure lipoxidase, low temperatures, and the homogeneous linoleate solution of low concentration used in our assay method. A preliminary report of these experiments has already appeared (18).

The oxygen uptake of the lipoxidase-oxidized linoleic acid was measured in the Warburg respirometer, using 3.2 ml. of substrate and a convenient quantity of enzyme (1.5–6.0 units) in 0.2 ml. under an atmosphere of air. The reactions were stopped by the addition of 5.0 ml. alcohol at appropriate oxygen absorptions, and the light absorption was measured at 234 and 270  $m\mu$  on these or diluted solutions, using the Beckman spectrophotometer. Plots of  $(\log I_0/I)/\text{molarity of linoleic acid}$  *vs.* degree of oxidation are shown in Fig. 6. The extinction coefficients, which are equal to the initial slopes, are high early in the reaction, and they decrease as the reaction proceeds, the magnitude of the decrease being dependent upon the temperature of the reaction mixture. The final two points in the 8°C. curve were made in an atmosphere of oxygen, and serve only to show the relatively high degree of conjugation possible even at a content of one mole of oxygen/mole acid. That the extinction coefficient for diene remains near 30,000 at 0°C., at least as far as 30% oxidation, is interpreted as indicating a complete conjugation of the monohydroperoxide formed from linoleic acid. The extremely high values at the very outset of the reaction are probably due to a lag in the measurement of oxygen. Statistical treatment (16) of the data obtained below 40% oxidation led to the most probable apparent extinction coefficients 31,400 at 0°C., 27,800 at 8°C., and

23,000 at 37°C. The apparent extinction coefficient under assay conditions (20°C. and oxygen) was found to be 25,600. The decrease in diene conjugation with increased temperature was paralleled by an increased absorption at 270 m $\mu$ , indicating that side reactions, further oxidation,

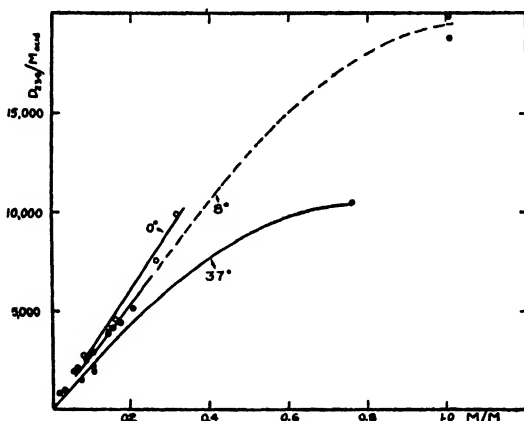


FIG. 6. Effect of temperature upon apparent extinction coefficient of products of lipoxidase oxidation of linoleic acid.

or decomposition of the peroxide are favored by the higher temperatures. See Fig. 7.

In the light of these observations, it is clear that under ideal conditions the removal of the hydrogen from (I) leads to a resonance hybrid composed of (II), (III), and (IV), to which oxygen adds only at the ends of the resonating system, leading to totally conjugated peroxides. It is suggested that the same mechanism applies to the autoxidation of linoleic and related acids because of the similarity of the enzymatic oxidations and autoxidations of these substances.

#### MOLECULAR ACTIVITY OF LIPOXIDASE

From the information now available concerning lipoxidase and its action, it is possible to estimate the substrate turnover of the enzyme. Using the maximum activity value of 850 units/mg., the products' apparent extinction coefficient of 25,600 at 20°C., and a molecular weight of lipoxidase of 102,000, the turnover value is 360 moles substrate/mole lipoxidase/second under our assay conditions. Because under assay conditions the enzyme is saturated with oxygen and at its

pH optimum, is nearly saturated with regard to substrate, and is near its temperature optimum, this turnover value approximates the enzyme's maximum possible activity. Inasmuch as the oxidation of linoleic acid, once initiated, can proceed *via* a chain reaction, it is impossible to say how much of this turnover is due to chain reactions and how much is due to direction action of lipoxidase.

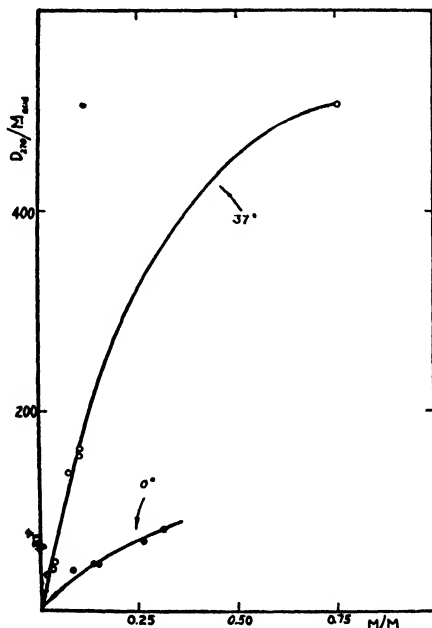


FIG. 7. Effect of temperature upon production of chromophores absorbing at 270  $m\mu$ .

#### ACKNOWLEDGMENT

The author wishes to express his appreciation to Professor Hugo Theorell for his advice in planning this investigation, and for the use of the facilities of the Medical Nobel Institute.

#### SUMMARY

The optimum pH for lipoxidase action lies near pH 9.4. Higher alkalinity does not markedly decrease the activity, but as neutrality is approached and emulsion systems are formed, the activity drops markedly.

The optimum temperature for lipoxidase is near 30°C. The  $Q_{10}$  between -1.5°C. and 18.5°C. is about 1.6.

Lipoxidase is not inhibited by pyrophosphate, fluoride, cyanide, azide, *p*-chloromercuribenzoate, mercury ions, or diethyldithiocarbamic acid. Thus, the active groups in lipoxidase are not heavy metals or sulfhydryl groups. Tocopherol and  $\alpha$ -naphthol act as inhibitors in the range of  $10^{-3}$  *M*.

Elaidolinolenic acid, 10,12-linoleic acid, oleic acid, and octanoic acid, arranged in decreasing order of competition, show affinity for the active center in lipoxidase.

No activator enzyme effect is demonstrable using crystalline lipoxidase and homogeneous substrate at pH 9.

The Michaelis constants for reaction of lipoxidase with substrate and oxygen are  $1.35 \times 10^{-3}$  *M* and  $4.2 \times 10^{-4}$  *M* respectively. Lipoxidase is saturated with respect to oxygen at 160 mm.  $O_2$ .

At low temperature, lipoxidase oxidation of linoleic acid in homogeneous substrate at pH 9 leads to the production of totally conjugated hydroperoxides of linoleic acid.

Under the conditions of the assay, lipoxidase oxidizes 360 moles substrate/mole lipoxidase/second.

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## Citrus Acetylerase

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Received July 25, 1947

### INTRODUCTION

Enzymes causing the hydrolysis of simple esters are found in all parts of the animal body, particularly in liver and in muscle tissue (1, 2). Bovine mammary gland contains diacetinase and tributyrinase activities, the latter of which can be preferentially heat-inactivated (3). The enzymes from these sources, as opposed to those from pancreas, hydrolyze the glycerides of the lower fatty acids and other simple esters but only hydrolyze those of the higher fatty acids poorly. No such enzyme has been previously reported in the plant kingdom, except possibly the "lipase" of wheat (4). Enzymes causing the hydrolysis of simple esters have, of course, been obtained from plant sources—for example, the enzyme in citrus fruit which hydrolyzes methyl butyrate (5). Characterization of these esterases has usually been limited to their action on a substrate or two.

This report deals with a previously uncharacterized type of plant enzyme, acetylerase. During an investigation of the specificity of citrus pectinesterase, it was found that a crude enzyme preparation hydrolyzed esters of acetic acid, whereas purified pectinesterase did not (6). From these observations it was evident that another esterase was present in citrus fruit. Since the enzyme hydrolyzed esters of acetic acid best, it was named acetylerase.

### METHODS

Acetylerase determinations were made by a procedure similar to that previously described for the determination of pectinesterase (7, 8). The rate of liberation of

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COOH groups was measured by continuous titration in the absence of added buffer. Routinely, 10 ml. of the desired kind and concentration of ester was pipetted into a reaction vessel supported in a constant-temperature bath at 30°C. Three ml. of 1 *N* NaCl<sup>3</sup> was added, and the pH of the mixture was brought to the desired value by the addition of *N*/50 NaOH after extensible electrodes had been placed in the solution. Water and enzyme were added to make a final volume of 20 ml. The reaction mixture was stirred by a stream of CO<sub>2</sub>-free air. Observations of the time and quantity of alkali required to maintain a constant pH were begun immediately. The amount of enzyme used was usually sufficient to require 1–3 ml. of *N*/50 NaOH in 10 minutes, 4–6 readings of time and alkali being made during this period. In all cases initial rates of hydrolysis were determined; these rates in no case extended beyond the region of linearity. When determinations were made at acid pH values where incomplete titration occurred, corrections were made in accordance with a titration curve for acetic acid. Likewise, determinations made at alkaline pH values were corrected for the non-enzymatic hydrolysis. No determinations are reported at alkaline pH values for which the alkali correction was greater than  $\frac{1}{4}$  of the enzymatic hydrolysis.

The results are reported in acetylerase units. One acetylerase unit is that quantity of enzyme which will cause the liberation of one milliequivalent of COOH groups per minute in 20 ml. of 5% triacetin at 30°C. in the presence of 0.15 *N* NaCl at pH 6.5. The symbols [AE.u.] g., [AE.u.] ml., and [AE.u.] mg. P.N. represent the milliequivalents of ester hydrolyzed/min./g., ml., or mg. of protein nitrogen of the enzyme under the conditions specified above. Where relative activities are reported, the activity value obtained under the above standard conditions was assigned the value of unity.

The solubility of monobutyryl in water was determined by saturating water with a large excess of monobutyryl (20 g./100 ml.). The excess monobutyryl was removed, and an aliquot of the solution was neutralized to phenolphthalein end-point, and subsequently saponified. From the saponification value the molarity was calculated and found to be 0.75 *M* (12.7%). This was approximately 15 times larger than that reported by Sobotka and Glick (9). However, it was found that a large excess of monobutyryl was necessary in order to obtain a saturated solution, since the monobutyryl apparently contained a water-insoluble impurity, and visual appearances alone were not sufficient to judge saturation. Since the method of Sobotka and Kahn (10) for the determination of solubility depends upon the appearance of a second phase as magnified by a dye, thus being based upon visual examination, it is felt that the value reported above for the solubility of monobutyryl in water is more nearly correct.

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<sup>3</sup> The NaCl had only a small activating effect on acetylerase as contrasted with its effect on the activity of pectinesterase (7, 8). However, the effect was sufficiently large to warrant the use of NaCl in routine assays.

## RESULTS

*Extraction of Acetylesterase.* During some early work on the preparation of acetylesterase from citrus flavedo by pressing, it was noted that the addition of small amounts of solid NaCl (1.5 g./100 g. of ground flavedo) increased the activity in the press juice. Furthermore, since the presence of salt was so essential for the extraction of pectinesterase from citrus tissue (8), all extraction experiments were carried out with 0.25 M NaCl solution as the extractant. The results reported in Table I show that extraction is essentially complete in one hour, regardless

TABLE I  
*Extraction<sup>a</sup> of Acetylesterase from Citrus Tissue*

Fruit	Natural pH (5.5)		pH 7	
	1 hr.	24 hr.	1 hr.	24 hr.
Grapefruit flavedo	1.2 <sup>b</sup>	1.0	1.4	1.4
Grapefruit albedo	0.7	0.3	0.8	0.8
Orange flavedo	1.5	0.8	2.1	2.2
Orange albedo	0.4	0.1	0.8	1.0

<sup>a</sup> To 50 g. of tissue was added 100 ml. of 0.25 N NaCl, followed by blending in a Waring Blendor, after which the mixture was stirred for 1 hour. Where the extractions were done at pH 7, alkali was added from time to time to keep the pH at 7 during the hour of stirring, after which little change occurred. After the desired time of extraction, the solid was filtered off with cheesecloth.

<sup>b</sup> All activities are [AE.u.] ml.  $\times$  1,000.

of pH, and that pH 7 is preferable over the natural acid pH of 5.5, since no apparent destruction of enzyme occurs with time at the higher pH.

*Distribution of Acetylesterase in Citrus Fruit.* It can be seen from Table II that the major portion of the acetylesterase occurs in the flavedo and decreases toward the center of the fruit. Like pectinesterase (8), it is associated with the solid material, little occurring in the juice. The decrease toward the center of the fruit, however, is in contrast with the results observed with pectinesterase where the pulp contains as much as, or more than, the flavedo,<sup>4</sup> but was similar, in general, to the distribution of citrus fruit phosphatase (11). Some phosphatase was found, however, to be in solution in the juice (11). Also, by way of

<sup>4</sup> Unpublished results.

contrast to pectinesterase (8), press juice from flavedo (Table II) contained considerable acetylerase. The amount of enzyme in the press juice could be increased by the addition of NaCl to ground flavedo before pressing. This addition, however, also increased the pectinesterase and hence, was detrimental in the preparation of pectinesterase-free acetylerase, which will be discussed later.

TABLE II  
*Distribution of Acetylerase in Citrus Fruit*

Fruit	Extracts of tissue				Filtered juice	Flavedo press juice <sup>a</sup>	
	Flavedo	Albedo	Pulp <sup>a</sup>	Juice cloud <sup>b</sup>		No addition <sup>c</sup>	NaCl added <sup>d</sup>
	[AE.u.] g. ×1000	[AE.u.] g. ×1000	[AE.u.] g. ×1000	[AE.u.] g. ×1000	[AE.u.] ml. ×1000	[AE.u.] ml. ×1000	[AE.u.] ml. ×1000
Oranges (Navel)	6.9	3.2	1.6	0.6	<0.02	3.8	7.3
Lemon	1.2	0.6	<0.02	—	<0.02	1.8	2.6
Grapefruit (Marsh Seedless)	4.2	2.5	0.9	—	<0.02	3.8	4.8

<sup>a</sup> The fruit was reamed and screened through cheesecloth. The material remaining on the cloth was designated pulp.

<sup>b</sup> The juice cloud was obtained by centrifuging screened juice.

<sup>c</sup> 100 g. of ground flavedo was mixed with 82 g. of sand and pressed with a Carver laboratory press, yielding approximately 50 ml. of press juice.

<sup>d</sup> 1.5 g. of NaCl was added to each 100 g. of flavedo during the grinding.

*Stabilization and Concentration of Acetylerase.* The first attempts to concentrate acetylerase either in an extract or press juice with  $(\text{NH}_4)_2\text{SO}_4$  resulted in 40–60% loss in activity and, as in most plant extracts, considerable darkening occurred during the process. In working with citrus pectinesterase, it was found that reducing agents like sulfite or monothioglycol would stabilize the enzyme during precipitation and dialysis.<sup>4</sup> Because of certain limitations and the undesirable odor of monothioglycol, other reagents were sought.

Sodium oxalate was found to prevent the darkening of flavedo press juices and even to reverse the coloration if it had not proceeded too far. Furthermore, saturation of a press juice with  $\text{Na}_2\text{C}_2\text{O}_4$  will cause no inactivation of the acetylerase. Approximately 90% of the acetylerase in orange and grapefruit press juices could be recovered in the precipitate formed by 0.7 saturation with  $(\text{NH}_4)_2\text{SO}_4$  at 0°C. of press juices saturated with  $\text{Na}_2\text{C}_2\text{O}_4$ . The non-darkened precipitate was taken up in a small volume of 0.1 M  $\text{Na}_2\text{C}_2\text{O}_4$ . The recovery with lemon flavedo press juice, how-

ever, varied between 30 and 90%. Dialysis of the concentrates against cold 0.1  $M$   $Na_2C_2O_4$  removed much of the pigment.

The following method for the preparation of a dialyzed, lyophilized orange flavedo press juice concentrate illustrates the manner of preparation of acetylcsterase from the various flavedos. These preparations were used in much of the subsequent work. To 1,440 g. of fresh orange flavedo was added 21.1 g. of solid  $NaCl$ , followed by grinding in a food grinder and mixing with 144 g. of Celite. The mixture was then pressed in a Carver laboratory press, using pressing cloth. The yield of press juice was 875 ml., and the juice had an activity of [AE.u.] ml. = 0.0053. (Pressing with Celite gave a higher yield of press juice than did pressing with sand, but gave only 90% as much total activity.) The press juice was then saturated with  $Na_2C_2O_4$ , which caused no change in activity. The solution was filtered clear and the enzyme precipitated by 0.7 saturation with  $(NH_4)_2SO_4$ . The precipitate was taken up in an amount of 0.1  $M$   $Na_2C_2O_4$  corresponding to 0.2 the volume of the original press juice. Over 90% of the activity was recovered. The concentrate was dialyzed against 0.1  $M$   $Na_2C_2O_4$  in the cold, with very little loss in activity. It was subsequently dialyzed against water in the cold which caused considerable precipitation and inactivation. The precipitate was extracted with water, combined with the dialyzate, and lyophilized. The overall recovery of enzyme (1.04 g. dry weight) was 41% of the original press-juice activity. The majority of the loss occurred during the water dialysis.

With grapefruit and lemon flavedo press juices, the yields were only 15 and 1%, respectively. The specific activities of these preparations are given in Table III. The specific activities on a mg. or a mg. nitrogen

TABLE III  
*Specific Activities of Dialyzed, Lyophilized Preparations of Acetylcsterase*

Flavedo	[AE.u.] mg.	[AE.u.] mg. N	[AE.u.] mg. PN
Orange	0.0015	0.018	0.024
Grapefruit	0.0006	0.006	0.008
Lemon	0.0002	0.004	0.018

basis were in proportion to the per cent yields of activity. On a mg. protein nitrogen basis, the specific activities were similar, particularly in the cases of the lemon and orange preparations. However, the specific activities of the preparations would suggest that they contained the order of 1% or less of acetylcsterase, as judged from the specific activity of pure enzymes (8).

*Preparation of Acetylcsterase with Low Pectinesterase Content.* Grapefruit flavedo (2,770 g.) was ground, mixed with sand (2,300 g.), and pressed in a Carver laboratory press.<sup>5</sup> The press juice was then fractionally precipitated with  $(NH_4)_2SO_4$ . As can be

<sup>5</sup> No  $NaCl$  was added here, since the amount of pectinesterase solubilized by the  $NaCl$  would greatly exceed the additional acetylcsterase that would be obtained.

TABLE IV  
*Partial Separation of Pectinesterase from Acetylerases<sup>a</sup>*

No.	Material	Concentration factor <sup>b</sup>	[PE.u.] ml.	Acetylerase		Ratio <sup>c</sup>
				[AE.u.] ml.	Per cent	
1	Press juice	1	0.0006	0.0014	100	2.3
2	Soln. of ppt. <sup>d</sup> obtained from No. 1 by 0.4 saturation with $(\text{NH}_4)_2\text{SO}_4$	5.23	0.0008	0.0047	62	5.9
3	Soln. of ppt. <sup>d</sup> obtained from the supernatant of No. 2 by increasing the $(\text{NH}_4)_2\text{SO}_4$ to 0.8 saturation	7.64	0.0017	0.0006	6	0.4
4	Oxalate-dialyzed No. 2 <sup>e</sup>	4.75	0.0003	0.0045	65	15

<sup>a</sup> Press juice from grapefruit flavedo was used as the source of the enzyme.

<sup>b</sup> The concentration factor represents the concentration on a volume basis, assigning the original press juice the value of one.

<sup>c</sup> Ratio = [AE.u.] ml. / [PE.u. ml.]

<sup>d</sup> The precipitates were taken up in 0.1 *M* sodium oxalate.

<sup>e</sup> The dialysis was carried out in the cold for 3 weeks against 0.1 *M* sodium oxalate.

seen from Table IV, 0.4 saturation of the press juice with  $(\text{NH}_4)_2\text{SO}_4$  at room temperature precipitated over 60% of the acetylerase and improved the ratio of acetylerase to pectinesterase from 2.3 to 5.9. The precipitate obtained between 0.4 and 0.8 saturation with  $(\text{NH}_4)_2\text{SO}_4$  contained mostly pectinesterase. Dialysis of a solution of the 0.4 saturation precipitate against 0.1 *M* sodium oxalate caused destruction of the pectinesterase without any loss of acetylerase. The final material contained, on an activity basis, 15 times as much acetylerase as pectinesterase. This solution kept well in the refrigerator for more than eight months.

*Effect of Salt on Acetylerase Activity.* As contrasted with the cation effect on the activity of citrus and other pectinesterases (7, 8), the activity of acetylerase is only slightly affected by salt. The results reported in Table V show the effect of 0.15 *M* NaCl at pH values of 5.5 and 6.5 on the dialyzed, lyophilized preparations of flavedo acetylerase. The greatest effect noted was the doubling of lemon acetylerase activity at pH 5.5 by the NaCl. At pH 6.5 the NaCl caused only about 20% increase in activity. This was, indeed, far removed from the 5- to 100-fold increase (dependent upon pH) caused by cations on the activity of citrus pectinesterase (8). At pH 4.5, salt had no effect on acetylerase activity. Therefore, in the light of the above

observations, acetylerase determinations were routinely run in the presence of 0.15 *M* NaCl.

TABLE V  
*Effect of NaCl on Acetylerase Activity*

Enzyme <sup>a</sup>		Relative activity			
Flavedo source	Amount per assay	pH 5.5		pH 6.5	
		No salt	0.15 <i>M</i> NaCl	No salt	0.15 <i>M</i> NaCl
Orange	4 mg.	0.76	0.96	0.78	1.0
Grapefruit	8 mg.	0.62	1.06	0.86	1.0
Lemon	7 mg.	0.43	0.93	0.61	1.0

<sup>a</sup> Dialyzed, lyophilized preparations of enzyme were used.

*Optimum pH for Acetylerase Action.* The pH optima for the action of acetylerase on mono-, di-, and triacetin was determined by the use of dialyzed, lyophilized enzyme, whose preparation is described above. The pH optima for the action of grapefruit acetylerase on the acetins are shown in Fig. 1. The optima were broad (between pH

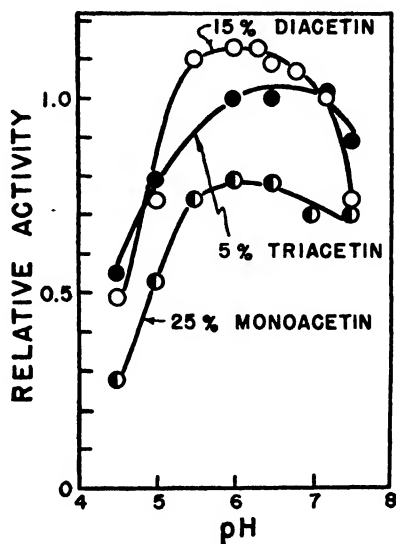


FIG. 1. The pH optimum for the action of grapefruit acetylerase on the acetins.

5.5 and 6.5), and were not markedly different for these three substrates. Similar results were obtained for the pH optima for the action of acetylsterase from the three sources on monoacetin (Fig. 2).

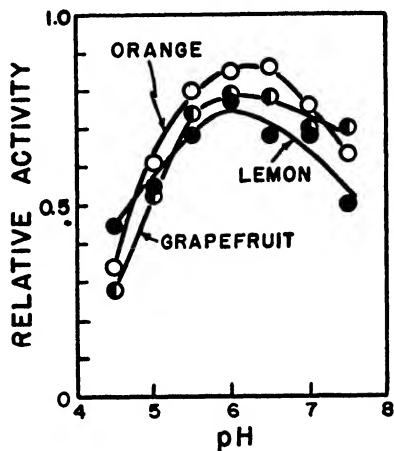


FIG. 2. The pH optimum for the action of orange, lemon, and grapefruit acetylsterase on monoacetin.

*Affinities ( $K_m$  values) and Maximum Velocities of Hydrolysis of Acetins, Acetyl-glycols, and Acetylcholine.* The action of an enzyme on a given substrate is characterized by two constants: the affinity or dissociation constant (Michaelis-Menten constant) of the enzyme-substrate complex, and a constant (maximum velocity) characteristic for the rate at which the end-products of the reaction are formed from this enzyme-substrate compound. It is only with a knowledge of both that true enzyme specificity can be determined. In certain cases, apparent enzyme specificity, as indicated from arbitrary substrate concentrations, would prove to be erroneous when the above constants are determined.

The Michaelis-Menten dissociation constant,  $K_m$ , is the substrate concentration where one-half of the theoretical maximum velocity occurs (a large  $K_m$  denotes small enzyme-substrate affinity). The  $K_m$  values were determined for the substrates, mono-, di-, and triacetin and mono- and diacetyleneglycol. The solid lines in Fig. 3 are the calculated curves obtained from the activity-substrate-concentration relationship following the treatment outlined by Lineweaver and Burk (12) for obtaining the maximum velocity ( $V_{max}$ ), using a dialyzed, lyophilized acetylsterase concentrate obtained from orange flavedo. The points are the observed relative activities. Although  $K_m$  for the various substrates, even on a normality basis, varied as much as 4 times, the  $V_{max}$  values varied only 30% in the extreme cases. Thus the theoretical maximum velocities for these substrates were practically the same, and the substrates differed only in the affinity of the enzyme for the particular ester. The  $K_m$  values reported here are 40–80 times greater than those of Sobotka and Glick (9) for the action of liver esterase on mono-, di-, and tributyrin.

Acetylcholine bromide, which was hydrolyzable by citrus acetylerase, had a  $K_m$  value of approximately  $1.6 M$  and a theoretical maximum velocity of approximately 2.4, which is considerably higher than that for the acetins (Fig. 3). Some idea of the high concentration necessary to realize maximum activity on this substrate may be gained when one considers that  $1.6 M$  is a 36.2% solution.

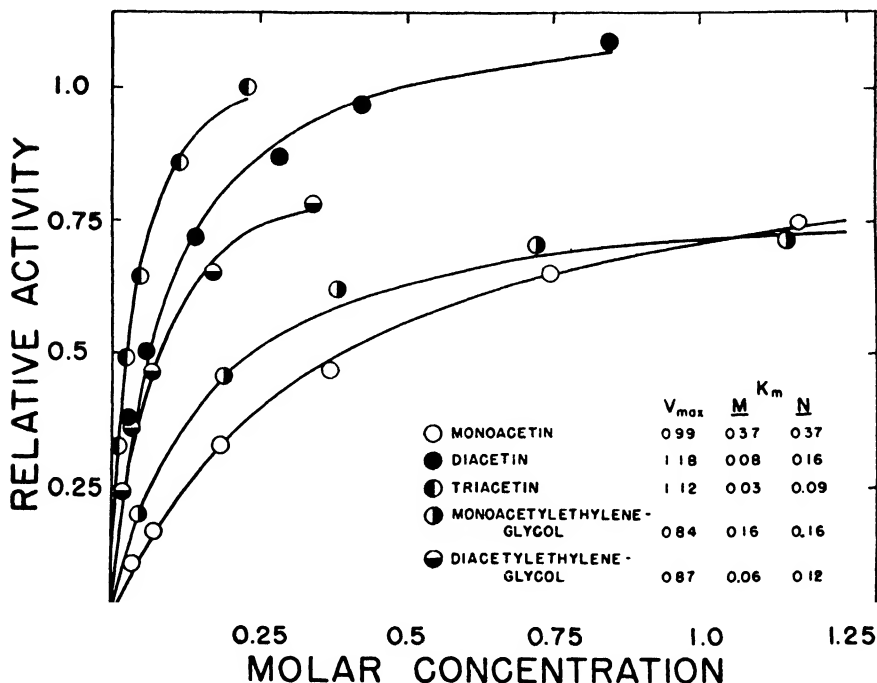


FIG. 3. The affinities and maximum velocities of acetylerase for several substrates. The points are experimental; the curves were calculated from the Michaelis-Menten equation for enzyme-substrate complex equilibrium.

It is apparent, therefore, that the affinity of citrus acetylerase for the substrates mentioned above is very small. To illustrate how misleading arbitrary substrate concentrations might be in the determination of enzyme specificity, the relative rates of hydrolysis of mono-, di-, and triacetin and acetylcholine bromide in 1% solution were found to be in a ratio of 1, 3.5, 4.3, and 0.13, respectively. As shown above, however, the theoretical maximum velocities for the acetins are roughly the same, and for the acetylcholine it is twice as large.

*Differentiation of Acetylerase from Cholinesterase.* In spite of the low affinity of acetylerase for acetylcholine bromide, it might be reasoned



that the enzyme is a cholinesterase merely because it hydrolyzes this substrate. Both true and pseudo cholinesterase of animal origin are inhibited by small concentrations of eserine, less than  $1 \times 10^{-6} M$  (13, 14). Eserine had no effect, however, on the action on acetylerase on acetylcholine bromide. This fact was shown in two ways: First, acetylerase was incubated with eserine at concentrations of  $1 \times 10^{-5}$  and  $10^{-4} M$  for 90 minutes and subsequently assayed, using acetylcholine bromide as the substrate. No decrease in activity was detected. Secondly, sufficient eserine was added to reaction mixtures of acetylerase and acetylcholine bromide to give concentrations of  $1 \times 10^{-5}$  and  $1 \times 10^{-4} M$ , but no inhibition was noted. Hence, by these criteria, acetylerase is not a cholinesterase.

*Differentiation of Acetylerase from Lipase.* The action of orange flavedo press juice on olive oil was determined by the use of the lipase method of Balls, Matlack and Tucker (15), adapted to a potentiometric titration at a constant pH of 8.0. For this purpose 4 ml. of orange flavedo press juice, equivalent to 0.02 AE.u., and 1 ml. of  $H_2O$  were added to 15 ml. of stock solution of olive oil emulsified in bile salts and 0.04  $M$  to  $CaCl_2$ , and the mixture was stirred with  $CO_2$ -free air. No hydrolysis was detectable in 20 minutes—that is, the addition of less than 0.1 ml. of 0.02  $N$  NaOH was necessary to maintain the pH at 8.0. By contrast, the action of this amount of enzyme on triacetin would have necessitated the addition of 20 ml. of 0.02  $N$  NaOH to maintain the pH at 6.5 during this time period. Thus the flavedo contained no typical lipase.

Although pancreatic lipase can readily hydrolyze emulsified fats, it might be reasoned that the citrus enzyme is a lipase and does not hydrolyze the higher glycerides because of their water insolubility, as Sullivan and Howe (4) suggested for the enzyme they obtained from wheat. Therefore, in order to ascertain further that acetylerase is not a lipase, the action of the enzyme was measured on water-soluble monoglycerides. The results reported in Table VI show that, on an

TABLE VI  
*Action of Acetylerase<sup>a</sup> on Water-Soluble Monoglycerides*

Monoglyceride	Relative activity	
	0.75 $M$ substrate <sup>b</sup>	0.375 $M$ substrate <sup>b</sup>
Monoacetin	0.53.	0.38
Monopropionin	0.19	0.28
Monobutylin	0.005	0.003

<sup>a</sup> 8.5 mg. of lyophilized acetylerase prepared from orange flavedo as shown alone was used for each 20 ml. of reaction mixture.

<sup>b</sup> These were the substrate concentrations in the final reaction mixture.

equimolar basis, monoacetin was hydrolyzed at a rate 100 times greater than monobutylin. Monopropionin was hydrolyzable at one-third to two-thirds the rate of monoacetin, depending on the concentration of the substrates. It is, therefore, obvious from the above results that the citrus enzyme is an esterase and not a lipase.

*Effect of Temperature and pH on the Stability of Acetylerase.* To avoid inactivation due to the reactions that occurred simultaneously with darkening (oxidation) of press juice, the oxalate-dialyzed grapefruit acetylerase, whose preparation is described above, was used to study the effect of temperature and pH on the enzyme. Fig. 4 illustrates

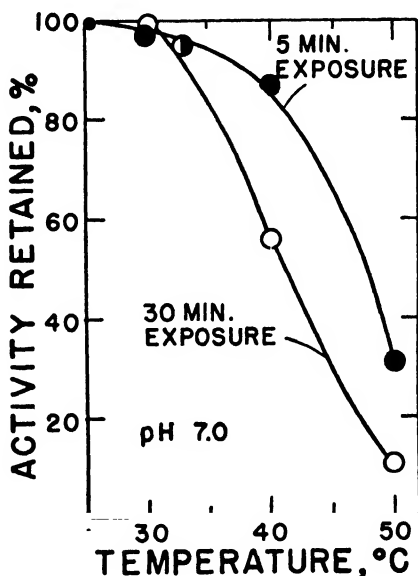


FIG. 4. The effect of temperature on the stability of acetylerase.

the stability of acetylerase as influenced by temperature. Temperatures above 35°C. inactivated the enzyme at pH 7.0, and at 50°C. the inactivation was rapid. This is in contrast with the stability of orange pectinesterase (8) when temperatures 10° higher were necessary to cause a corresponding inactivation.

Fig. 5 indicates the effect of pH on acetylerase with time at 25°C. The enzyme is quite unstable below pH 4. At these low pH values the inactivation was rapid, most of the loss having occurred within the

first hour. At pH values of 5.0 and 8.25, however, the enzyme was remarkably stable; approximately 50% of the activity remained after 6 days.

*Specificity of Acetylesterase.* The action of acetylesterase on several esters of acetic acid is shown in Table VII. The acetylesterase prepara-

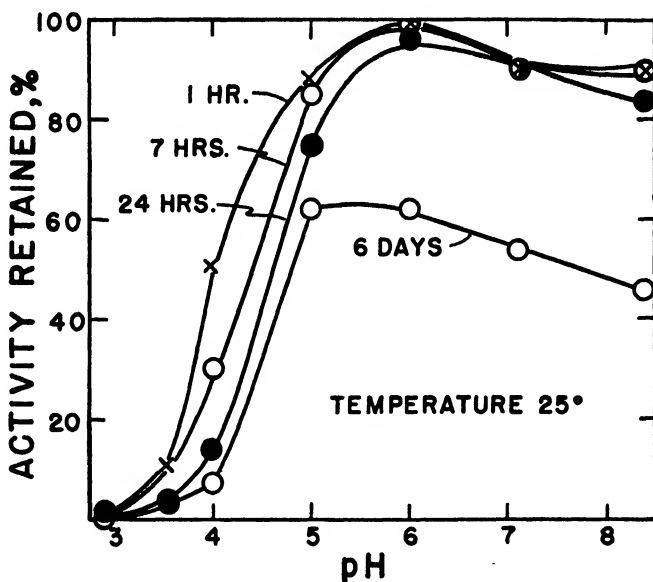


FIG. 5. Effect of pH on the stability of acetylesterase.

tion low in pectinesterase (as described above) was used in this study. All the esters of acetic acid were hydrolyzable and, at optimum substrate concentration, the rates were 0.2–0.4 of that observed on triacetin. Even substrates of low solubility such as the sugar acetates were hydrolyzed at a fairly rapid rate.

The results obtained with other esters are shown in Table VIII. Methyl and ethyl butyrate were hydrolyzed at a rate only 0.1–0.2 of that observed on triacetin, whereas tripropionin and ethyleneglycol diformate were hydrolyzed at the rates of 0.3 and 0.5, respectively. Tributyrin hydrolysis was very slow; the relative rate was only 0.04. The rate observed on methyl butyrate, when calculated to equal basis, was only one-sixth of that observed by Sosa and Sannie (5). The anhydride, acetyl phosphate (11), was not hydrolyzable by acetylester-

ase.<sup>6</sup> The hydrolysis, if any, of N-acetyethanolamine by acetylerase was less than 1% of that of triacetin when measured either by the method described above or by the formol titration method. No hydrolysis of N-acetylglucosamine could be detected by the latter method, or of acetyl-*o*-aminophenol by the former method. Hence, it is apparent that acetylerase is a specific esterase whose activity is greatest on esters of acetic acid, but is not active on substituted acetamides or anhydrides involving an acetyl moiety.

TABLE VII

*Action of Acetylerase<sup>a</sup> on Several Esters of Acetic Acid*

Substrate	Concentration per cent	Relative activity
Methyl acetate	0.5	0.08 <sup>b</sup>
	2.5	0.29 <sup>b</sup>
	5.0	0.42 <sup>b</sup>
	10.0	0.24 <sup>b</sup>
Ethyl acetate	0.5	0.03 <sup>b</sup>
	2.5	0.16 <sup>b</sup>
	5.0	0.24 <sup>b</sup>
	10.0	0.13 <sup>b</sup>
Allyl acetate	0.5	0.05
	1.0	0.20
	1.5	0.30
	2.5 <sup>c</sup>	0.25
Benzyl acetate	0.5 <sup>c</sup>	0.25
	1.0 <sup>c</sup>	0.32
Xylitol pentacetate	0.5 <sup>c</sup>	0.22
Tetraacetylmethyl- $\alpha$ -D-galacturonate	0.5 <sup>c</sup>	0.20

<sup>a</sup> The grapefruit acetylerase preparation low in pectinesterase was used in these experiments.

<sup>b</sup> These values were based upon initial activities. The activities decreased rapidly with time.

<sup>c</sup> These substrates were not completely soluble at the concentrations indicated.

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<sup>6</sup> In conjunction with Dr. B. Axelrod of the Enzyme Research Laboratory, it was demonstrated that the acetylphosphatase activity of citrus fruit was proportional to the nitrophenylphosphatase activity (11). Several enzyme preparations which had varied ratios of acetylerase and phosphatase were used to establish this proportionality.

## DISCUSSION

Acetylesterase has been shown to be a typical esterase having its greatest activities on esters of acetic acid. The activity on the glycerides decreased as the chain length of the fatty acids increased, even with the water-soluble monoglycerides. This decrease was very marked. The glycerides of butyric acid were hydrolyzed at a rate less than 4%

TABLE VIII  
*Action of Acetylesterase on Several Esters*

Substrate	Concentration	Enzyme preparation	Relative activity
	<i>per cent</i>		
Methyl- <i>n</i> -butyrate	0.5	Grapefruit—low in pectinesterase	0.12 <sup>a</sup>
	1.0	Grapefruit—low in pectinesterase	0.14 <sup>a</sup>
	2.0	Grapefruit—low in pectinesterase	0.13
	2.5	Grapefruit—low in pectinesterase	0.16
Ethyl- <i>n</i> -butyrate	0.5	Grapefruit—low in pectinesterase	0.09 <sup>a</sup>
	1.0 <sup>b</sup>	Grapefruit—low in pectinesterase	0.13
Ethyl acetoacetate	5.0	Orange flavedo press juice	0.06
Acetyl- <i>o</i> -aminophenol	1.0	Orange flavedo press juice	0.00
Ethyleneglycol diformate	5.0	Lyophilized orange	0.48
Tripropionin	10.0 <sup>b</sup>	Grapefruit flavedo press juice	0.30
Methyl propionate	10.0 <sup>b</sup>	Grapefruit flavedo press juice	0.07
Tributylin	1.0 <sup>b</sup>	Grapefruit flavedo press juice	0.04

<sup>a</sup> These values were based upon initial activities. The activities decreased rapidly with time.

<sup>b</sup> These substrates were not completely soluble at the concentrations indicated.

of that of the acetins. These results suggest that other plant enzymes which have been classified as lipase—for example, the “lipase” of wheat (4)—may in reality be a similar esterase.

An enzyme with the characteristics of acetylesterase has never previously been reported to occur in the plant kingdom. The role it plays in plant metabolism is obscure. The possibility that it functions as an essence- or flavor-synthesizing enzyme is intriguing, since esters of acetic acid have been found in citrus fruit.

The facts that the predominance of the acetylesterase occurs in the flavedo and decreases toward the center of citrus fruit, that the enzyme

is unstable below pH 3.5, and that it is easily inactivated by heat, as opposed to pectinesterase (8), make it doubtful that the enzyme would interfere with the keeping quality of processed citrus products.

The stabilization of acetylerase by oxalate was apparently due to the prevention of oxidative darkening of the accompanying materials. This observation may well be applicable to the stabilization of the other plant proteins. The darkening of alfalfa press juice which had been freed of chloroplasts was prevented by oxalate.<sup>4</sup>

#### ACKNOWLEDGMENTS

We are indebted to Dr. Hans Lineweaver of the Western Regional Research Laboratory for many helpful suggestions during the course of this work, and to Dr. Bernard Axelrod of the Enzyme Research Laboratory for his cooperation in establishing the correlation of acetylphosphatase activity with phosphatase content, rather than the acetylerase contents of several citrus enzyme preparations.

#### SUMMARY

1. Acetylerase, an enzyme that hydrolyzes esters of acetic acid best, occurs in oranges, lemons, and grapefruit. Salt solution (0.25 *N* NaCl) at pH 7.0 was used for extraction of the enzyme from citrus tissue.

2. The predominance of the acetylerase occurred in the flavedo and decreased toward the center of the fruit. This distribution was in contrast to that of pectinesterase but similar to that of phosphatase.

3. Sodium oxalate was found to stabilize the enzyme during such manipulations as ammonium sulfate precipitation and dialysis. The oxalate prevented darkening of the enzyme solutions, which may have been the reason for its stabilizing effect.

4. A preparation of acetylerase with a low pectinesterase content was obtained by fractional precipitation of grapefruit flavedo press juice with  $(\text{NH}_4)_2\text{SO}_4$ , followed by dialysis against 0.1 *M* sodium oxalate. This preparation contained, on an activity basis, 15 times as much acetylerase as pectinesterase.

5. Salts had only a small activating effect on acetylerase, which was in contrast to the effect of cations on citrus pectinesterase. The greatest increase in activity caused by sodium chloride was only 2-fold.

6. The pH optima for the action of the acetylerase from the various citrus fruits on the acetins were broad, and occurred between pH 5.5 and pH 6.5.

7. The affinities, the Michaelis-Menten dissociation constants,  $K_m$ , of acetylerase for the acetins, the acetylglcols, and acetylcholine were determined, and the maximum velocities of hydrolysis,  $V_{max.}$ , were calculated from the activity-substrate-concentration relationship. The theoretical maximum velocities for the acetins and the acetylglcols were practically the same. However, the affinities of the enzyme for the various substrates differed and were considerably lower than those usually observed. The  $K_m$  value for acetylcholine bromide was even higher than that found for the acetins. However, the  $V_{max.}$  was more than twice that obtained for the acetins.

8. Although acetylerase hydrolyzes acetylcholine, the enzyme was shown not to be a cholinesterase by the failure of eserine to cause inhibition.

9. Acetylerase was shown not to be a lipase. The rate of hydrolysis of tributyrin was only 4% of that of triacetin. No hydrolysis of olive oil was detectable by preparations of acetylerase. A study of the rates of hydrolysis of the water-soluble monoglycerides in equimolar concentrations showed that the failure to hydrolyze the higher glycerides was not due merely to water insolubility. Monobutyryl was hydrolyzed at a rate of only 1% of that of monoacetin, while that of monopropionin was intermediate. The enzyme is therefore an esterase and not a lipase.

10. Temperatures above 35°C. inactivated the enzyme at pH 7.0, and at 50°C. the inactivation was rapid. This fact was in contrast to the stability of orange pectinesterase, when temperatures 10° higher were necessary to cause a corresponding inactivation. The enzyme was found to be unstable below pH 4.0. An enzyme so easily inactivated would hardly be expected to contribute to the deterioration of processed citrus products.

11. A study of the specificity of acetylerase showed that all aliphatic esters of acetic acid were hydrolyzed by the enzyme. N-acetyl-ethanolamine, N-acetylglucosamine, and acetyl phosphate were not hydrolyzable. Other simple esters such as methyl and ethyl butyrate and ethyleneglycol diformate were hydrolyzed, but at a slower rate than were the acetins.

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# The Microbiological Determination of Glutamine in Human Plasma

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Received July 28, 1947

## INTRODUCTION

Glutamine has been determined by the ninhydrin carbon dioxide method (1), by measurement of the ammonia liberated by acid (2) or enzymatic treatment (3), and by estimation of glutamic acid before and after conversion of glutamine to the acid by an acid hydrolysis (4). It is well known that glutamine or glutamic acid is required for the growth of many lactic acid bacteria. The requirement for these two substances in the growth of nine lactic acid bacteria was studied by Pollack and Lindner (5). The organisms studied by them were capable of utilizing glutamine with apparently maximum efficiency but varied considerably in their response to glutamic acid. However, many organisms can utilize glutamic acid and glutamine interchangeably which makes it difficult to distinguish glutamine and glutamic acid microbiologically. Thus Prescott and Waelsch (4) point out, "microbiological methods are not applicable at present to the determination of glutamic acid in the presence of glutamine."

The observation of McIlwain (6) that ammonium pyrrolidone- $\alpha$ -carboxylate was not effective as a growth stimulant was confirmed for *Lactobacillus arabinosus* 17-5 by Lyman *et al.* (7). Hamilton (1) made a thorough study of the reaction by which glutamine is converted to pyrrolidone carboxylic acid without affecting the glutamic acid present as determined by the ninhydrin carbon dioxide method, and in this communication (1) he stated that glutamine is readily converted, practically quantitatively, by heating in solution under the conditions defined with regard to pH and time, to pyrrolidone carboxylic acid. Hac, Snell and Williams (8) in a study of the requirements of *L. arabinosus* for glutamic acid and glutamine utilized the same principle in the development of a microbiological assay method for glutamic acid. These authors also showed that the activity of glutamic acid for this organism could be increased to substantially that of glutamine by increasing the size of the inoculum, lengthening the incubation period, lowering the initial pH of the basal medium and

adding ammonium salts, presumably to facilitate conversion of glutamic acid to glutamine, since their data indicated that this reaction must occur before glutamic acid is utilized. These authors further suggested that it should be possible to determine glutamine and glutamic acid microbiologically by a refinement of the technique reported in their paper.

The present study reports the development of a method for the microbiological assay of glutamine in the presence of glutamic acid and its use in the assay of human blood plasma, employing as a fundamental reaction the conversion of glutamine to pyrrolidone carboxylic acid under the conditions specified by Hamilton (1) for glutamine assay by a gasometric technique.

### EXPERIMENTAL

All microbiological assays were carried out using a basal medium essentially the same as that described by Schweigert *et al.* (9) for the assay of valine and leucine with *L. arabinosus* 17-5 (8014) except that glutamic acid was omitted. In accordance with the suggestions of Hac, Snell and Williams (8), 1.2 g. of  $(\text{NH}_4)_2\text{SO}_4$ /100 cc. of basal medium were added, the pH was adjusted to 6.3 before use, and a heavy inoculum was employed. With this technique, reproducible curves similar for added increments of 10-50  $\gamma$  of glutamic acid or glutamine were obtained and, hence, glutamic acid was used as a standard for all assays. One cc. of basal medium was added to each assay tube with supplements to a total volume of 2 cc. The acid produced after a 72-hour incubation period was measured by titration with *N*/50 NaOH with the aid of a Beckman meter.

A study of the inactivation of glutamine by conversion to pyrrolidone carboxylic acid, as detected by microbiological assay, was made by comparing the behavior of a solution of L-glutamic acid with that of a solution containing glutamic acid and L-glutamine in equal amounts when both were made 0.08 *m* with  $\text{KH}_2\text{PO}_4$  (10.9 mg./cc.), the pH adjusted to 6.5, and heated at 100°C. for periods up to 150 minutes. Aliquots of the original solutions were sterilized by Seitz filtration and added aseptically to assay tubes after these had been heat-sterilized and cooled. Other samples were assayed after autoclaving but before being heated to 100°C., and finally, while being heated, samples were removed for assay at 30 minute intervals. Table I shows the changes in glutamic acid (or glutamic acid plus glutamine) concentration with time. It is apparent that, under these conditions, the glutamic acid concentration remained unchanged although the glutamine was converted to a microbiologically unavailable form (pyrrolidone carboxylic acid) in a period of 60-90 minutes. There was a rapid conversion in the first period, and a considerable part of the reaction occurred during autoclaving (10 lbs. 10 minutes) prior to inoculation.

Assays of human blood plasma were carried out by the following method:

The plasma was deproteinized by dilution with water (1:1), the mixture being placed in a boiling water bath for one minute; 1 drop of 5% acetic acid per cc. of plasma originally taken was then added and the tube was replaced in the water bath for about one minute. The proteins were promptly precipitated and after centrifuga-

tion a water-clear solution was obtained. In recovery experiments in which glutamine was added to plasma before this heating procedure, there was no detectable loss of glutamine as a result of this short heating period. Hamilton (1) points out that

TABLE I

*Microbiological Determination of the Concentration of Glutamine and Glutamic Acid after Heating for Various Periods in 0.08 m KH<sub>2</sub>PO<sub>4</sub> at pH 6.5*

Solution A was a mixture of 25  $\gamma$  L-glutamine and 25  $\gamma$  L-glutamic acid/cc.; Solution B contained 25  $\gamma$  L-glutamic acid/cc. Each value is an average of 2 tubes.

Sample	Before auto-claving	After auto-claving	Heated at 100°C. in .08 m KH <sub>2</sub> PO <sub>4</sub> , pH 6.5				
			30 min.	60 min.	90 min.	120 min.	150 min.
A	50	34	28	26	$\gamma$ /cc. 25	26	25
B	25	24	26	25	25	24	26

glutamine is relatively stable to heat when acetate is the buffer. The centrifugate was decanted and made 0.08 m in KH<sub>2</sub>PO<sub>4</sub> by adding the solid compound in appropriate quantity, and the pH was adjusted to 6.5. Usually one drop of N/1 NaOH sufficed. Thus, the volume change due to these added reagents was negligible. One portion was sterilized by filtration through a Seitz filter and another portion was heated in a boiling water bath for 90 minutes. The heated samples were added to the basal medium before sterilization in the autoclave but the unheated, Seitz sterilized samples were added aseptically to the medium after sterilization and cooling. This was necessary because, as was shown above, autoclaving destroyed a part of the glutamine present. Assays were made at different levels but 1 cc. samples of the heated fraction equivalent to 0.5 cc. of the original plasma, and 0.4 and 0.6 cc. samples of the unheated fraction were found most convenient. The activity of the unheated portions was taken to represent the total glutamine and glutamic acid concentration of the filtrate. The residual activity after heating was interpreted as a measure of the glutamic acid concentration of the original sample.

The apparent free glutamine and glutamic acid content of human plasma as determined by this procedure is shown in Table II. These assays were carried out on the plasma from heparinized blood obtained from 10 normal healthy males after a 12-hour fast. The results are very similar to those previously reported for plasma both in man and other animals when determined by various chemical methods (1, 2, 3, 4, 10).

## DISCUSSION

Hier and Bergeim (11) reported finding less than 6  $\gamma$  of free glutamic acid/cc. in six samples of dog plasma which they assayed micro-

TABLE II  
*Apparent Free Glutamine and Glutamic Acid in Normal  
 Human Plasma after a 12 Hour Fast*

Subject	Glutamine glutamic acid (before heating)	Glutamic acid (after heating)	Difference (glutamine)
		<i>mg./100 cc. plasma</i>	
Fg.	6.9	0.5	6.4
P.	11.0	1.4	9.6
Hb.	6.1	>0.5	
T	13.0	1.4	11.6
S.	11.7*	0.8	10.9
W.	10.5	0.8	9.7.
Ha.	8.3	>0.5	
Fr.	8.2	0.6	7.6
Hv.	10.2	0.8	9.4
M.	10.5	0.8	9.7

biologically. In the 10 human samples here reported, 7 showed an activity attributable to glutamic acid of 6  $\gamma$ /cc. or more, but only 2 had more than 8  $\gamma$ /cc. Since the sensitivity of the method for glutamic acid is limited when samples contain less than 5  $\gamma$ , it is conceivable that free glutamic acid would appear to be virtually absent from many samples of plasma when examined microbiologically unless larger quantities of sample are taken for assay. The observation of previous workers that the great majority of glutamic acid-like compounds in the plasma is actually glutamine is confirmed and, in view of the heat-labile nature of glutamine, assays carried out after autoclaving would show little or no activity other than that due to the glutamic acid originally present.

Unna and Howe (12) and Madden (13) reported that nausea and vomiting were observed when glutamic acid was injected intravenously. Similar symptoms have sometimes been noted when casein hydrolyzates are administered, giving rise to the suggestion that glutamic acid is the offender since its concentration in these hydrolyzates is relatively high. The very small quantities of free glutamic acid in fasting human plasma indicate that, as in the bacterial cell, glutamic acid is in large part converted to glutamine before being further metabolized. The untoward symptoms observed after intravenous glutamic acid administration might then be attributable to the fact that the acid is

presented to the tissues at a rate which exceeds their ability to perform this conversion to glutamine.

Dunn *et al.* (14) reported that the D-isomer of glutamic acid was partially active for *L. arabinosus* 17-5 (8014) but that this isomer had no activity for *Streptococcus faecalis* R (8043). They listed seven other organisms which would respond to both the D- and L-isomers. In the course of the work here reported, it was noted that *Strep. faecalis* R and *Lactobacillus lyopersici* (4005) respond to glutamine as well as glutamic acid but do not utilize pyrrolidone carboxylic acid. This last organism is one of those recommended by Dunn *et al.* (14) for the determination of DL-glutamic acid. Thus, it would appear that the method for glutamine assay described in this communication could be used with these organisms where it was desirable to distinguish between the enantiomorphs.

#### ACKNOWLEDGMENTS

The blood samples assayed were obtained from the U. S. Naval Hospital, Oakland, California, through the courtesy of Drs. Laurance W. Kinsell and Harry C. Barton.

The gift of a sample of L-glutamine from General Biochemicals, Inc., Chagrin Falls, Ohio, is gratefully acknowledged.

Pyrrolidone carboxylic acid was prepared in the Chemical Laboratory of the University of San Francisco by Mr. Richard Burke and kindly made available for these experiments by Dr. Joseph V. Karabinos.

#### SUMMARY

1. A microbiological method for the determination of glutamine in the presence of glutamic acid has been developed.

2. Analysis of 10 normal human plasma filtrates for glutamine by this method yielded fasting values ranging from 6.4 to 11.6 mg./100 cc. These are in good agreement with those previously reported after chemical assay.

3. The high ratio of glutamine to glutamic acid in human plasma is confirmed.

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# The Lipotropic Activity of Caffeine, Theobromine and Theophylline

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Received July 30, 1947

## INTRODUCTION

Evidence has been available for some time that the methylated purines are at least partially demethylated in the animal body. The older German literature is summarized in Cushny's textbook (1). Myers and Hanzal (2) first showed that caffeine and theophylline are oxidized and partially demethylated in the animal. Bernheim and Bernheim (3) studied the process *in vitro*. They found that liver slices of rabbit, guinea pig and rat were able to oxidize caffeine and theophylline. Theophylline was probably partially demethylated but experimental limitations made it impossible to decide whether caffeine was demethylated by their preparations.

If caffeine, theobromine and theophylline can be demethylated, the question arises whether this process can effectively contribute to the animal's requirements for labile methyl groups. Moyer and du Vigneaud (4) found that caffeine did not permit growth of rats on a methionine- and choline-free diet containing homocystine. Mills and Cottingham (5) observed that caffeine protected against hemorrhagic nephritis in rats due to choline deficiency but it was somewhat less effective than methionine. They also followed the growth of rats in simulated tropical environments. Under these conditions the requirements of choline for growth were increased. Methionine seemed well able to replace choline in supporting growth, but with caffeine in place of choline growth was retarded.

In the present study male weanling rats were fed a low protein-high fat diet deficient in choline. Some of the animals received dietary sup-



plements of caffeine, theobromine or theophylline. It was observed that all three compounds were lipotropic.

### EXPERIMENTAL

Male weanling rats of the Sprague-Dawley strain were fed a diet of the following composition: purified casein,<sup>1</sup> 6.0; hydrogenated cottonseed oil, <sup>2</sup> 38.0; U.S.P. cod liver oil, 5.0; salts,<sup>3</sup> 2.0; and cane sugar, 49.0. The diet also contained in mg./kg.: thiamine hydrochloride, 10; calcium pantothenate, 40; nicotinic acid, 40; pyridoxine

TABLE I

*Influence of Caffeine, Theobromine and Theophylline on Liver Fat Content of Rats Fed Choline-Deficient Diet*

Group	No. of rats	Dietary treatment	Initial body weight (average)	Final body weight (average)	Liver weight (average)	Liver fat content (average)
Experiment 1						
I	10	Basal diet for 21 days	g. 46.5±1.4 <sup>a</sup>	g. 45.5±1.7	g. 4.12±0.22	per cent 28.4±1.5
II	8	Basal diet for 27 days	45.6±1.1	46.3±2.0	4.02±0.28	29.3±2.9
III	13	0.3% Caffeine for 21 days	48.1±1.6	39.6±1.8	2.24±0.09	11.9±1.1
IV	11	0.3% Caffeine for 27 days	49.9±1.7	40.4±1.5	2.66±0.16	17.3±1.8
V	7	0.3% Caffeine for 13 days, then 0.4% caffeine for 8 days.	45.9±2.2	36.1±2.0	2.04±0.09	9.5±1.0
VI	5	0.3% Caffeine for 13 days, then 0.4% caffeine for 8 days, then 0.6% caffeine for 7 days.	46.0±1.7	34.6±1.7	2.16±0.13	13.5±2.3
Experiment 2						
VII	10	Basal diet for 26 days	35.4±1.1	38.6±1.1	3.93±0.23	32.3±2.2
VIII	10	0.4% Theobromine 16 days, then 0.6% theobromine for 10 days.	35.9±0.9	32.0±1.3	2.11±0.31	12.4±1.1
IX	8 <sup>b</sup>	0.4% Theophylline for 26 days.	34.6±1.5	27.4±1.4	1.14±0.26	12.6±1.6

<sup>a</sup> Including the standard error of the mean calculated as  $\sqrt{d^2/N - 1} / \sqrt{N}$ .

<sup>b</sup> 10 Rats were begun on experiment of which 2 died after 6 and 7 days.

<sup>1</sup> General Brands, Inc.

<sup>2</sup> Crisco.

<sup>3</sup> Hubbell, Mendel and Wakeman (6).

hydrochloride, 10; riboflavin, 20; biotin, 0.01; 2-methyl-1,4-naphthoquinone, 4. Once weekly the rats received 3 mg. of  $\alpha$ -tocopherol dissolved in 0.03 ml. ethyl laurate. Some of the animals obtained supplements of caffeine, theobromine or theophylline mixed in their diet. The compounds were commercial preparations that had been recrystallized. The rats were kept in individual metabolism cages.

In Expt. 1 (Table I) the rats were divided into 6 groups. Group I received the basal diet for 21 days, after which the rats were killed and their livers analyzed for total fat. Group II was fed the basal diet for 27 days. Groups III and IV received 0.3% caffeine in their diet for 21 and 27 days respectively. The diet of group V contained 0.3% caffeine during the first 13 days on test. The rats were then given 0.4% caffeine for 8 days. Group VI received 0.3% caffeine in the diet for 13 days, then 0.4% caffeine for 8 days and finally 0.6% caffeine for 7 days. The level was increased stepwise in the hope that the rats would develop some tolerance to the drug. Fifteen litters of rats were used for the experiment. One member of each litter was placed on the basal ration while the others received the supplemented diets.

In Expt. 2 (Table I) 30 rats were divided into 3 groups, equal with respect to litter distribution. Group VII was fed the basal choline-deficient diet for 26 days. Group VIII received a dietary supplement of 0.4% theobromine for 16 days and 0.6% during the next 10 days. Group IX was fed 0.4% theophylline for 26 days.

The livers were weighed and analyzed for total fat by the method of Artom and Fishman (7). This involves preliminary extraction with a mixture of hot alcohol and ether. After evaporation nearly to dryness the alcohol-ether extractable material is reextracted with hot chloroform and finally dried and weighed.

## RESULTS

The content of total chloroform-soluble material in the livers of the rats is shown in Table I. The differences in liver fat content between the groups on the basal diet and those fed supplements were analyzed by Fisher's *t*-method (8) and found to be significant ( $p < 0.01$ ). In the present study no comparison was made between the lipotropic activity of the methylated purines and methionine. But in connection with another investigation 20 male weanling rats of the same strain were kept for 23 days on the basal diet described in this paper supplemented with 1% methionine. This is equivalent in total methyl content to 0.47% caffeine. The rats ate an average of 2.6 g./day and their liver fat content was  $10.5 \pm 0.9\%$ . Eighteen unsupplemented rats also ate 2.6 g./day and showed a liver fat content of  $38.9 \pm 0.7\%$ .

Table II gives the average daily food consumption for the rats in the present study. It is evident that food intake was much poorer for the group fed theophylline than for the others. The rats given theophylline appeared nervous and restless. Two of them died, after 6 and 7 days on the diet. The animals fed the other two supplements showed no evidence of intoxication, except for loss in weight.

TABLE II

*Average Daily Food Consumption of Young Rats on a Basal Choline-Deficient Diet and with Various Supplements Added to the Diet*

Supplement	Content in diet	Average daily food consumption
	Experiment 1	
	<i>per cent</i>	<i>g.</i>
None	—	2.9±0.1
Caffeine	0.3	2.6±0.1
Caffeine	0.4	2.6±0.5
Caffeine	0.6	2.5±0.3
	Experiment 2	
None	—	2.5±0.1
Theobromine	0.4	2.8±0.2
Theobromine	0.6	2.6±0.1
Theophylline	0.4	1.8±0.1

### DISCUSSION

These experiments show that caffeine, theophylline and theobromine have lipotropic activity. This is presumably due to the fact that these compounds can be demethylated and make possible choline synthesis in the rat. However, it is conceivable that some peculiar pharmacological property of the compounds may be responsible for the effect observed. Thus, Hindemith (9) has stated that caffeine increased the unsaturation of liver fatty acids.

In a single feeding trial we confirmed Moyer and du Vigneaud (4), who found that caffeine did not stimulate growth when added to a methionine- and choline-free diet containing homocystine. Another instance of a compound which prevented fatty livers and hemorrhagic kidneys in rats, and yet was relatively inefficient in substituting for choline on methyl-free diets, was dimethylaminoethanol (10).

### ACKNOWLEDGMENT

Miss Louise Odor assisted in carrying out some of these experiments.

### SUMMARY

A low casein-high fat diet, deficient in choline, was fed to male weanling rats for several weeks. Some of the animals received supplements of caffeine, theobromine or theophylline. The content of these methylated purines in the diet varied from 0.3 to 0.6%. At the con-

clusion of the feeding period the animals were killed and their livers were analyzed for total chloroform-soluble substances. All three compounds were found to be lipotropic.

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## Note on the Ultraviolet Absorption Spectra of Keto Acids and of Keto Acid Peptides

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Received July 31, 1947

Studies on the spectral characteristics in the ultraviolet of pyruvic acid and its peptide with glycine, namely, pyruvoylglycine, revealed (1) that, in solutions at pH < 10, the former possessed an absorption maximum at 3300 Å, while the latter possessed absorption maxima at 3400 Å and 2400 Å, (2) that in solutions of pH > 10, the absorption maxima for both compounds disappeared, the rate of disappearance for pyruvic acid being nearly instantaneous, that for pyruvoylglycine being more gradual, and (3) that when the pH of the alkaline solution was brought back to less than 10, the original absorption curve of pyruvic acid was restored, whereas the curve for pyruvoylglycine did not change (1, 2). Further, chemical evidence that the alkalization of pyruvoylglycine was irreversible came through the observations (1) that, after bringing to pH > 10, the capacity of the keto acid peptide to form the 2,4-dinitrophenylhydrazone (m.p. 242°C.) was lost, and (2) that, whereas treatment with hot HCl of originally acid or neutral solutions yielded nearly quantitative recoveries of pyruvic acid and glycine, similar treatment of the previously alkalized solution yielded neither pyruvic acid nor  $\alpha$ -amino nitrogen (1, 2). On the basis of these data, plus the fact that the alkalized compound, like the original, possessed a pK 3.3, the phenomena were interpreted as due to a molecular rearrangement of the enolic form of the keto acid peptide in alkaline solution with ring closure to form a  $\gamma$ -hydroxypyrrolidone carboxylic acid.

It was considered desirable to extend this study to an analogous pair of compounds, and phenylpyruvic acid and phenylpyruvoylglycine were chosen for this purpose. Phenylpyruvic acid was prepared by the

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hydrolysis of acetaminocinnamic acid (3), and phenylpyruvoylglycine by the hydrolysis of acetaminocinnamoylglycine (4; *cf.* 5). Both compounds readily dissolve to clear solutions in water at pH 7.0 following addition of dilute NaOH.

The spectra of the keto acids and of their peptides in aqueous solution at pH 7.0, obtained by the use of the Beckman quartz spectrophotometer, are given in Fig. 1. The continuous absorption of the com-

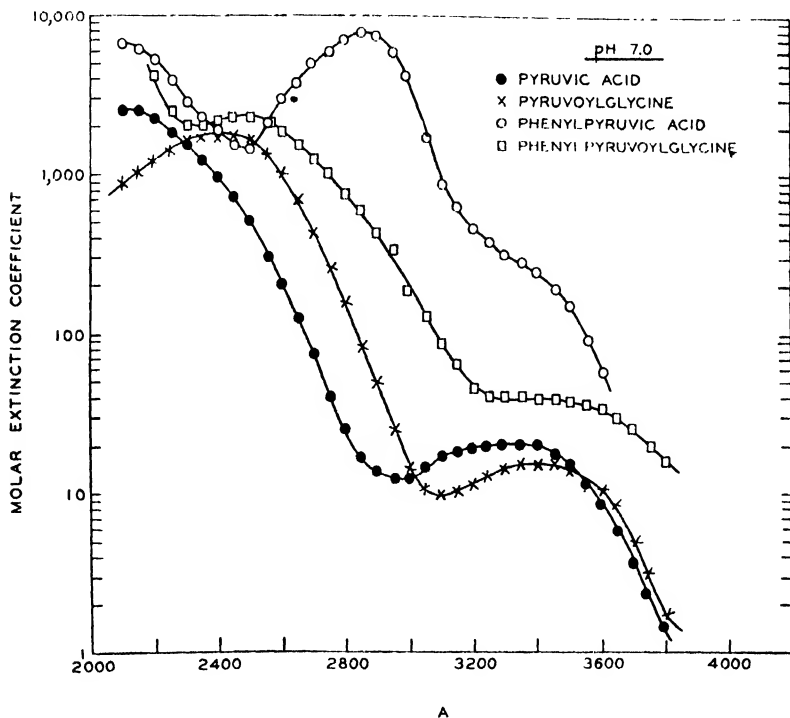


FIG. 1. Ultraviolet absorption spectra of keto acids and keto acid peptides in water at pH 7.0. Concentration  $2.27 \times 10^{-4} M$ .

pounds in the region of 3200–3500 Å is attributable to the presence of carbonyl groups (6). Phenylpyruvic acid possesses an absorption band at 2850 Å, phenylpyruvoylglycine possesses a band at 2500 Å. The absorption bands in the region 2500–2900 Å for the keto acids with aromatic rings may be due to the influence of the phenyl substituents, but it is difficult to apprehend the reason for the presence of the strong band at 2400 Å for pyruvoylglycine (Fig. 1).

When dilute NaOH was added at 25°C. to the neutral solutions of phenylpyruvic acid and of phenylpyruvoylglycine to bring the pH to 11.5, the absorption spectra of these compounds almost instantly altered into the forms given in Fig. 2. No further change occurs for several hours in the shape of the curves. In such alkaline solutions, the curves for both phenylpyruvic acid and phenylpyruvoylglycine possess

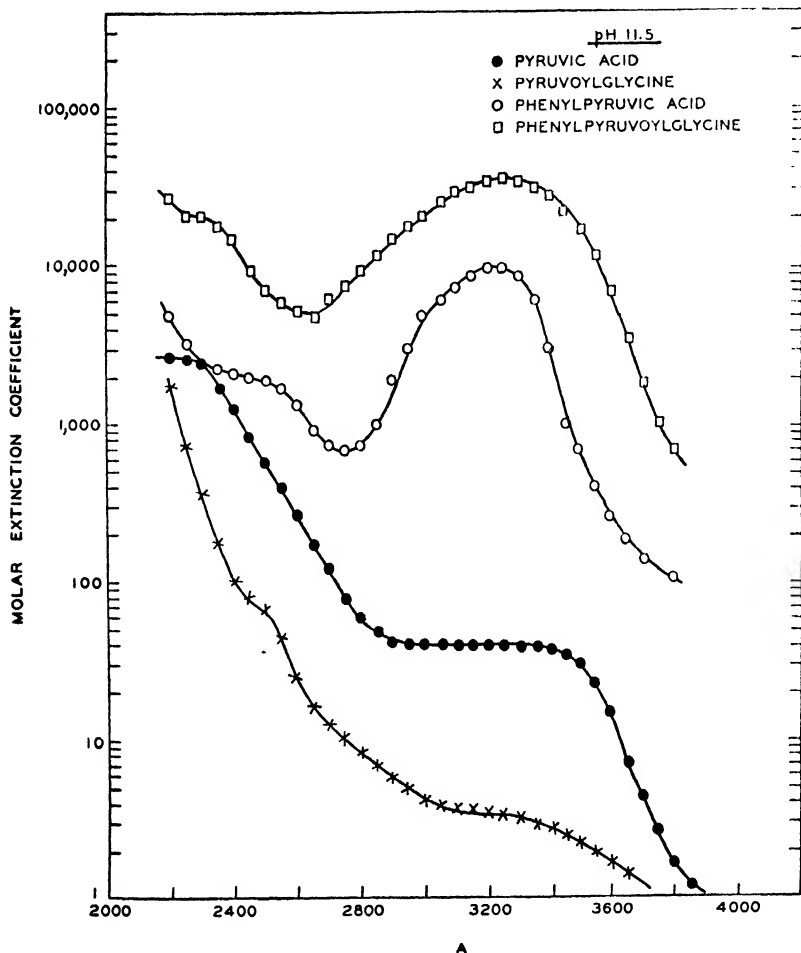


FIG. 2. Ultraviolet absorption spectra of keto acids and keto acid peptides in water at pH 11.5. Concentration of phenylpyruvoylglycine  $2.27 \times 10^{-5} M$ . All others at  $2.27 \times 10^{-4} M$ .



well-marked absorption bands with a maximum for both compounds at 3250 Å. Pyruvic acid under similar conditions shows continuous absorption in this region, while pyruvoylglycine absorbs very little (Fig. 2).

When the alkaline solutions of phenylpyruvic acid and of phenylpyruvoylglycine are carefully brought back to pH 7.0 by addition of dilute HCl, the absorption spectra of both compounds revert instantly and almost completely to the original spectra shown in Fig. 1. Furthermore, both compounds readily form the respective 2,4-dinitrophenylhydrazones which possess absorption spectra in the visible region qualitatively and quantitatively identical with those of compounds which had never been previously alkalized (*cf.* 2, 5).

The alkanization, therefore, of pyruvic acid, of phenylpyruvic acid, and of phenylpyruvoylglycine, is reversible, whereas that of pyruvoylglycine is irreversible. It is probable that the irreversible changes which readily occur in the molecular configuration of pyruvoylglycine may be spatially hindered from occurring in phenylpyruvoylglycine by the presence of the benzene ring.

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# Effect of Various Keto Acids on the Desamidation of Glutamine

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Received July 31, 1947

## INTRODUCTION

The accelerating effect of added pyruvic acid on the rate of desamidation of glutamine and of asparagine in aqueous extracts of rat liver has been described (1-5). This effect is apparently independent of glutaminase or asparaginase activity (6), and the ammonia which appears as a result of this effect can be quantitatively accounted for by a decrease in the amide nitrogen of the glutamine (1, 2). The present study is concerned with the effect of other keto acids on the desamidation of glutamine at various concentrations of the acids, and in the case of pyruvic acid, at two different concentrations of glutamine. For further purposes of comparison, lactic acid was also employed. At the pH of 6.8 used, the activity of glutaminase in rat liver extracts is negligible (1, 6).

## EXPERIMENTAL PROCEDURE

### *Materials and Methods*

The glutamine was a gift from the American Cyanamide Company, and on 1 minute hydrolysis with hot HCl yielded 98% of the theory of amide nitrogen. Pyruvoylglycine (1, 7) and phenylpyruvoylglycine (8) were prepared as described in the literature. In the preparation of the latter compound, it was noted that in the final step a brief period of boiling in 1 N HCl was necessary to hydrolyze the acetyl group from the acetylcinnamoylglycine. Phenylpyruvic acid was prepared from acetaminocinnamic acid (9).  $\alpha$ -Ketoglutaric acid was a gift from Dr. P. P. Cohen of the University of Wisconsin. The levulinic acid was an Eastman product. Glycyldehydroalanine (10) and glycyldehydrophenylalanine (11) were prepared as described. Fresh prep-

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arations of phenylpyruvic acid and of phenylpyruvoylglycine were employed because of the reported instability of these compounds (*cf.* 9). With very pure preparations kept in the vacuum desiccator over  $P_2O_5$  no change in the melting point or ultraviolet absorption spectra of these compounds could be observed over several weeks, nor in their effect on glutamine desamidation in rat liver digests.

The enzymatic determinations were performed with digests consisting of 1 cc. aqueous rat liver extract equivalent to 333 mg. fresh tissue, plus 1 cc. veronal acetate buffer at pH 6.8, plus 1 cc. of glutamine solution, plus 1 cc. of keto acid solution brought to neutral reaction with dilute NaOH. Glutaminase activity was determined by substituting 1 cc. of water for the keto acid solution, and the value for the extract blank was determined by substituting 2 cc. of water for the glutamine and keto acid solutions. The period of incubation was 4 hours at 37°C. At the end of this time, the pH of the digests was 6.7–6.8. The desamidation of the glutamine was measured by the amount of ammonia produced in the digests over that found in the controls. As noted previously, the ammonia so produced is accounted for by a corresponding decrease in the amide nitrogen of the glutamine (1, 2). Under the experimental conditions used, the  $\alpha$ -amino group of glutamine or of the glutamic acid subsequently formed, yields little or no free ammonia nitrogen (1).

The effect of the various keto acids on the desamidation of glutamine is described in Fig. 1. Of all keto acids studied, pyruvic acid produces

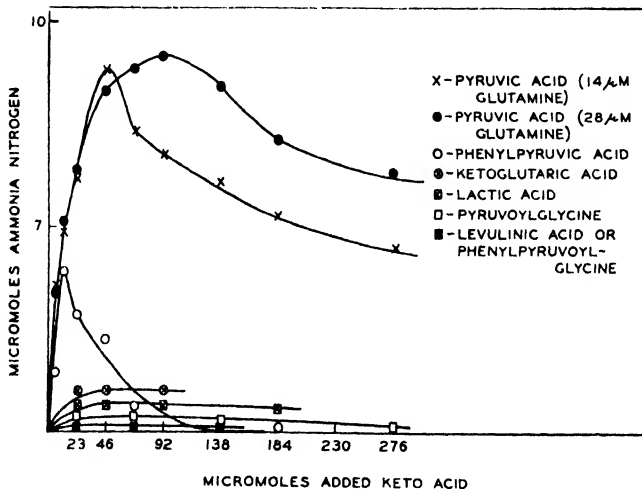


FIG. 1. Effect of added keto acids and lactic acid on the desamidation of glutamine at pH 6.8 in rat liver extracts. Ordinate refers to increment in ammonia nitrogen split from glutamine in presence of added keto and hydroxy acids over that in absence of these added acids. Ammonia N from digests of glutamine alone usually < 0.5 micromole. Except where designated, glutamine present to 14 micromoles. Incubation period 4 hours at 37°C.

the greatest effect in increasing the desamidation of glutamine, while phenylpyruvic acid produces a smaller, but still quite appreciable, effect. The effect of these two keto acids reaches a maximum with increasing amounts, for pyruvic acid at a ratio of about 3 moles per mole of glutamine (at both concentrations of glutamine studied), and for phenylpyruvic acid at a ratio of about 1 mole per mole of glutamine.

$\alpha$ -Ketoglutaric acid, pyruvoylglycine, phenylpyruvoylglycine, levulinic acid, and lactic acid increase the desamidation of glutamine only very slightly.

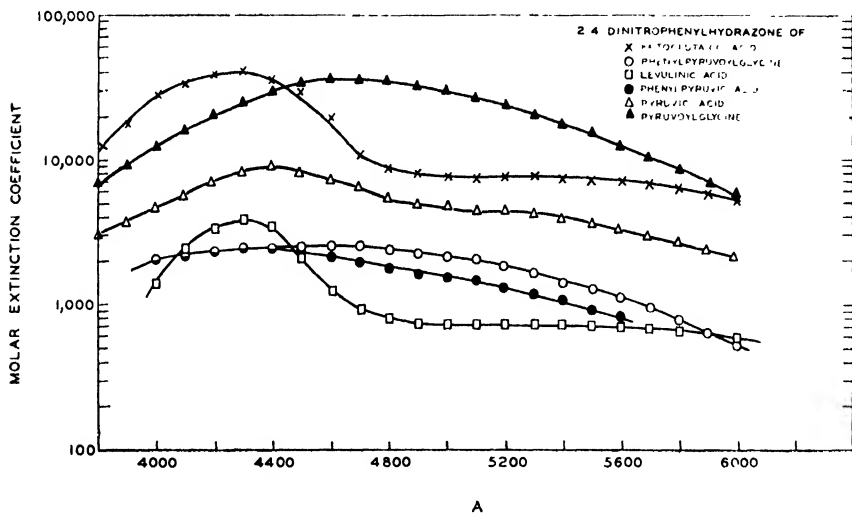


FIG. 2. Absorption spectra of 2,4-dinitrophenylhydrazones of several keto acids and their peptides with glycine.

It had been demonstrated that at the end of the incubation period of digests of rat liver with glutamine plus pyruvic acid, the pyruvic acid could be recovered in an amount nearly identical with that recovered from similar rat liver digests with added keto acid alone, or from 80–90% of the amount of pyruvic acid originally added (1, 2). Whatever role the pyruvic acid plays in the acceleration of glutamine desamidation, it is apparently not consumed in the reaction to any appreciable extent. To see whether the other keto acids could be recovered after digestion in the presence of glutamine, the absorption curves of their 2,4-dinitrophenylhydrazones were determined at pH 12 in the visible region of the spectrum (12) (Fig. 2) by the Beckman spectro-

photometer. The curves for the 2,4-dinitrophenylhydrazones of pyruvic acid and of pyruvoylglycine have been reported by the authors (12).

The absorption curves for the 2,4-dinitrophenylhydrazones of pyruvic acid, ketoglutaric acid, and levulinic acid possess well-defined maxima at 4300 Å. That for pyruvoylglycine possesses a well-defined maximum at 4600 Å. The corresponding curves for phenylpyruvic acid and for phenylpyruvoylglycine are somewhat similar (Fig. 2) and suggest an ill-defined maximum region of absorption for the former of 4200–4500 Å, and for the latter of 4400–4700 Å.

These curves were used to identify the hydrazones obtained after digestion of the keto acids with glutamine. The 2,4-dinitrophenylhydrazones were recovered by the method described (13), and the complete absorption curves compared qualitatively and quantitatively with those obtained at the same original concentration in water. In every case, the absorption curve of the keto acid dinitrophenylhydrazone after the digestion period resembled closely that of the pure compound, and was recovered to the extent of 80–90% of the amount originally added. The contribution by the keto acids native to the liver extracts was negligible compared with the amount of keto acid added.

Since increasingly large amounts of added pyruvic acid appeared to diminish the accelerating effect on the desamidation of glutamine (Fig. 1), and since the tentative explanation advanced for the mechan-

TABLE I  
*Inhibition of Dehydropeptidase I Activity in Aqueous Rat Liver  
Extracts by Added Pyruvic Acid*

Added pyruvic acid $\mu M$	Ammonia N from 23 micromoles glycyldehydroalanine $\mu M$	Per cent inhibition
0	7.0	—
12	7.0	0
23	6.8	3
46	6.7	5
92	6.1	13
184	4.9	26
276	3.8	38
542	3.3	53

Digests consisted of 1 cc. aqueous rat liver extract equivalent to 333 mg. fresh tissue, plus 1 cc. veronal acetate buffer at pH 6.8, plus 1 cc. glycyldehydroalanine solution containing 23 micromoles substrate, plus 1 cc. of either water or sodium pyruvate solution at concentrations designated. Period of incubation one hour at 5°C.

All solutions brought to this temperature before mixing. Data given corrected for extract blanks. pH of all digests at end of experiment 6.7–6.8.

ism of the effect of pyruvic acid on this desamidation involved the action of dehydropeptidase (1), it was considered of interest to note the effect of such increasing amounts of added pyruvic acid on the splitting of a known dehydropeptide, namely glycyldehydroalanine (Table I). This substrate is catalytically hydrolyzed in all animal and plant tissues to glycine, ammonia, and pyruvic acid (14), and the inhibitory action of added pyruvic acid may possibly be ascribed to a mass action effect.

The effect on dehydropeptidase activity by other keto acids, using not only glycyldehydroalanine but also glycyldehydrophenylalanine as substrate, is described in Table II.

TABLE II  
*Effect of Keto Acids and Keto Acid Peptides on the  
Hydrolysis of Dehydropeptides*

Keto acid or keto acid peptide added:	Ammonia evolved from:		Per cent inhibition of the splitting of:	
	Glycyldehydro- alanine	Glycyldehydro- phenylalanine	Glycyldehydro- alanine	Glycyldehydro- phenylalanine
—	$\mu M$	$\mu M$	—	—
Pyruvic acid	6.3	1.3	—	—
Phenylpyruvic acid	4.2	0.0	34	100
Pyruvoylglycine	1.2	0.9	81	31
Phenylpyruvoylglycine	6.0	1.2	5	7
Phenylpyruvoylglycine	6.0	1.2	5	7

Digests consisted of 1 cc. aqueous rat liver extract equivalent to 333 mg. fresh tissue, plus 1 cc. veronal acetate buffer at pH 6.8, plus 1 cc. of dehydropeptide solution containing 23 micromoles substrate, plus 1 cc. of either water or keto acid solution containing 276 micromoles. All keto acid solutions brought to pH 7.0 with dilute NaOH prior to use. Period of incubation when glycyldehydroalanine was used 1 hour at 5°C; when glycyldehydrophenylalanine was used 2 hours at 37°C. Data given corrected for extract blanks. pH of all digests at end of experiment 6.7–6.8.

On hydrolysis of glycyldehydrophenylalanine, glycine, ammonia, and phenylpyruvic acid are obtained (11). The data in Table II show: (1) that both pyruvic acid and phenylpyruvic acid inhibit the splitting of glycyldehydroalanine and of glycyldehydrophenylalanine, and (2) that, in contrast, neither pyruvoylglycine nor phenylpyruvoylglycine,

at the same concentrations and at the same pH as the free keto acids, significantly inhibits the splitting of the dehydropeptides. In several experiments on the effect of the keto acids on the splitting of glycyldihydroalanine, phenylpyruvic acid was invariably more inhibitory than was pyruvic acid. Because of the relatively low rate of enzymatic hydrolysis in liver homogenates of glycyldihydrophenylalanine, the difference in inhibitory effect of pyruvic acid and phenylpyruvic acid is not always as clear-cut and as considerable as revealed by the data in Table II. At this time, we wish only to emphasize the fact that the keto acid peptides do not significantly inhibit the enzymatic splitting of dehydropeptides under conditions whereby the free keto acids inhibit appreciably.

In view of these findings, the effect was studied of adding the same amounts of the keto acids and of their peptides to digests containing glutamine and a suboptimal quantity of pyruvic acid (Fig. 1). The data are given in Table III. The experiments with excess pyruvic acid

TABLE III

*Influence of Added Keto Acids and Keto Acid Peptides on the Pyruvate Effect on Glutamine Desamidation in Rat Liver Digests*

Keto acid or keto acid peptide added	Ammonia N evolved: $\mu M$	Per cent inhibition
—	95	—
Pyruvic acid	70	26
Phenylpyruvic acid	5	95
Pyruvoylglycine	65	31
Phenylpyruvoylglycine	58	38

Digests consisted of 1 cc. aqueous rat liver extract equivalent to 333 mg. fresh tissue, plus 1 cc. veronal acetate buffer at pH 6.8, plus 1 cc. of glutamine solution containing 14 micromoles, plus 1 cc. pyruvic acid solution containing 23 micromoles, plus 1 cc. of either water or keto acid solution containing 276 micromoles. All keto acid solutions were brought to pH 7.0 with dilute NaOH prior to use. Data given are corrected for extract blanks. Final pH of all digests 6.7–6.8.

were, of course, repetitive with those described in Fig. 1. However, phenylpyruvic acid to a considerable degree, and the keto acid peptides to an appreciable degree, also inhibited the usually accelerating effect of the pyruvic acid concentration used on the desamidation of glutamine.

## DISCUSSION.

Of all the keto acids studied, pyruvic acid produces the most considerable effect on the desamidation of glutamine. Whereas 14 micromoles of glutamine incubated under the present conditions with rat liver extracts yield less than 0.5 micromole of ammonia N, the same amount of glutamine in the presence of 46 micromoles of pyruvic acid yields about 9 micromoles of ammonia N (Fig. 1). With 28 micromoles of glutamine in the digest the maximum effect of added pyruvic acid is reached with 92 micromoles of the keto acid. Beyond these maxima, further addition of pyruvic acid produces less and less of an increase in glutamine desamidation. That this property of pyruvic acid is due essentially to the presence of the carbonyl group is shown by the relatively small effect of lactic acid.

Whether the keto acids studied increase or fail to increase the desamidation of glutamine in the rat liver digests, they nevertheless can all be equally well recovered at the end of the digestion period. In the case of those digests containing the keto acids which yield small amounts of ammonia N from glutamine (ketoglutaric acid, pyruvoylglycine, *etc.*), such apparently negative results might have been due to the possible taking up of ammonia from glutamine by the keto acids. The finding that all the keto acids can be nearly quantitatively recovered disposes of this possibility, and the inference is secure that such keto acids simply have little or no effect on the desamidation of glutamine.

The accelerating effect of pyruvic acid on the desamidation of amino acid amides is apparently confined only to glutamine, asparagine, and to chloroacetylglutamine, since the desamidation of isoglutamine and peptides of asparagine is not affected by this keto acid (3). This effect, furthermore, is noted only in extracts of liver, and not in those of any other mammalian tissue studied (3). In analogy with the ready, synthetic condensation *in vitro* of amides and keto acids to form dehydropeptides (10), the biological effect on glutamine and asparagine by pyruvic acid has been interpreted as being due to two consecutive enzymatic reactions: (1) a condensation between the amide group of the amino acid amide with the carbonyl group of the pyruvic acid to form the corresponding dehydropeptide, followed by (2) the hydrolysis of the dehydropeptide by dehydropeptidase to form the amino acid,



ammonia, and the regenerated pyruvic acid (1):



The curves in Fig. 1 relating to pyruvic acid and phenylpyruvic acid may be a composite of two curves. The ascending portion may be due to the increased rate of condensation of pyruvic acid or phenylpyruvic acid with glutamine as the keto acid concentration is increased up to a point where the large amount of added keto acid produces such an inhibition of dehydropeptidase activity (Tables I and II) as to overcome its accelerating effect on the initial condensation reaction, with the result that the curve begins to descend. On this basis, the keto acid peptides, which do not accelerate the desamidation of glutamine appreciably (Fig. 1), do not condense with the glutamine, nor do they inhibit dehydropeptidase activity (Table II). They can, however (Table III), interfere with the acceleration of the desamidation of glutamine by pyruvic acid, perhaps by a form of competitive inhibition with the pyruvic acid at the surface of the enzyme responsible for the condensation reaction.

### SUMMARY

Several keto acids in varying concentrations were incubated at pH 6.8 with glutamine in the presence of aqueous rat liver extracts. Only pyruvic acid, and, to a lesser extent, phenylpyruvic acid, produced an appreciable increase in the desamidation of glutamine.  $\alpha$ -Ketoglutaric acid, pyruvoylglycine, phenylpyruvoylglycine, and levulinic acid increased the desamidation of glutamine slightly if at all. At the end of the incubation period, each of the keto acids used could be nearly quantitatively recovered as the 2,4-dinitrophenylhydrazones. The absorption spectra of the various keto acid dinitrophenylhydrazones are described.

With increasing concentrations of pyruvic acid and phenylpyruvic acid, the desamidation of glutamine is increased to a maximum which, for pyruvic acid, appears to be at a ratio of about 3 moles of keto acid to 1 mole of glutamine, and, for phenylpyruvic acid, of about 1 mole of keto acid to 1 mole of glutamine. At higher ratios, the effect of the keto acid diminishes.

In contrast with the effect of pyruvic acid, lactic acid has little effect on the desamidation of glutamine.

At a relatively high concentration, pyruvic acid and phenylpyruvic acid appreciably inhibit the enzymatic hydrolysis of glycyldehydroalanine and of glycyldehydrophenylalanine, whereas, under the same conditions, pyruvoylglycine and phenylpyruvoylglycine possess little or no inhibitory capacity. All four keto acids, however, when added in excess, inhibit the accelerating effect of smaller quantities of pyruvic acid on the desamidation of glutamine.

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# The Phenylalanine and Tryptophan Content of Meats<sup>1</sup>

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Received August 14, 1947

## INTRODUCTION

The valine, leucine, and isoleucine content of meats based on microbiological determinations with *Lactobacillus arabinosus* has been reported by Schweigert *et al.* (1). Since this organism can be used to measure tryptophan quantitatively, and since enzymatic methods for the liberation of tryptophan have been described (2), studies have been extended to include this amino acid. *L. arabinosus* can also be used for the analysis of phenylalanine in animal tissues. Values for these two amino acids in meats and the retention of these amino acids during the various cooking procedures are presented in this paper.

## EXPERIMENTAL

The procedure which was used for the enzymatic hydrolysis and the microbiological determination of tryptophan is described in a previous publication (2). The medium and the methods of assay for phenylalanine are the same as those which were used in the assay for tryptophan with the appropriate amino acid omitted from the medium. A sample of DL-phenylalanine was used for the standard curve and tubes were set up in triplicate at the following levels: 0, 4, 10, and 14  $\gamma$  and in sextuplicate at 8, 12, 16, and 20  $\gamma$  per tube. In one group of assays the total final volume was 10 ml. of assay mixture per tube, and in a second group of assays the total final volume was 2 ml. When the total final volume was 10 ml. the blank titration was 0.8 ml. of 0.1 *N* sodium hydroxide, while the titration of the acid produced in the tube containing 20  $\gamma$ . of DL-phenylalanine was 14.0 ml. Comparable results were obtained when the total volume was 2 ml. The incubation period in both cases was 3 days.

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<sup>1</sup>Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the National Live Stock and Meat Board.

Previous work on leucine, valine (Schweigert *et al.*, 3), and isoleucine (Schweigert *et al.*, 1) showed that after the samples were autoclaved for 5 hours with 2 *N* hydrochloric acid they were satisfactorily hydrolyzed for the determination of those amino acids. The present work showed that a 5–10 hour period was required for the maximum liberation of phenylalanine when 2 *N* hydrochloric acid was used. Sulfuric acid did not give any greater liberation of the amino acid. Stronger concentration of acid did not result in higher values, so 2 *N* hydrochloric acid was used in all subsequent assays. Two different levels of samples were used, 1 g. was autoclaved with 25 ml. and 10 g. were autoclaved with 200 ml. of 2 *N* hydrochloric acid for 5 hours, at 15 lbs. pressure. After the solutions were autoclaved they were cooled and diluted to 100 and 500 ml., respectively, and suitable aliquots were neutralized and further diluted to 100 ml. and duplicate aliquots at three levels were used for assay. When DL-phenylalanine was added to samples of meat, recoveries of 99–107% of the added phenylalanine were obtained.

The preparation of the meat samples for the determination of tryptophan was reported earlier (2). The samples were tested using 2 ml. total assay mixture on one group of meats and 10 ml. for a second series of meat samples.

The meat samples were standard cuts from representative portions of the animals. They were ground and thoroughly mixed using sanitary techniques. These samples were used while fresh and the remaining portions of meats were stored at  $-4^{\circ}\text{C}$ . in dark colored bottles. The method of cooking the meat samples has been reported earlier (Schweigert *et al.*, 4).

TABLE I  
*Variation in Consecutive Assays of the Phenylalanine of Veal*

Assay No.	1	2	3	4	5	6	Value accepted
Sample	—	—	—	—	—	—	—
1	—	*4.0	3.8	4.0	3.6	3.9	3.9
2	3.9	4.2	4.0	3.9	3.9	4.0	4.0
3	4.0	3.8	3.7	3.9	3.9	4.0	3.9
4	4.3	4.2				3.9	4.1
5	3.9	4.0				3.8	3.9
6	4.0	4.3				3.7	Reject

\* Per cent in the protein (calculated to 16% N).

In Table I are given individual analyses for phenylalanine. Six assays were run on representative samples from each type of meat and 3 assays were run on all of the samples. Further investigation was made if more than 1 of the 6 samples showed a variation greater than 0.4% phenylalanine. Thus, the lowest value for veal sample No. 1 was 3.6% and the highest was 4.0%. Since all the samples were within the 0.4% range this sample was not analyzed further. If the individual samples in the 3 assays of the remaining samples did not fall within a 0.4% range or if they did, but the average of the 3 assays was not within 0.4% of the other values for similar cuts of meats, they were tested further. Sample No. 6, shown in Table I, was analyzed further since the 3 values were not within 0.4% of each other.

The percentages of tryptophan and phenylalanine in fresh and cooked muscle tissues and in beef organs are shown in Table II. The percentages of these amino acids in the proteins (calculated on the basis of 16% N) are given also. On the basis of the change in weight during the cooking process the retention of these amino acids during the cooking process was calculated (Schweigert *et al.*, 5). The retention of phenylalanine during cooking was 91–110% and of tryptophan was 73–96%.

TABLE II  
*Analysis of Meats*

Cut of meat	Protein	Phenylalanine			Tryptophan		
		In meat	In protein	Retention	In meat	In protein	Retention
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
<b>Veal</b>							
Shank	21.7	0.85	3.9		0.28	1.3	
Shoulder	20.4	0.82	4.0		0.31	1.5	
Roast shoulder	27.4	1.07	3.9	100	0.38	1.4	91
Loin	17.1	0.70	4.1		0.29	1.7	
Round	20.4	0.80	3.9		0.30	1.5	
Roast round	29.0	1.10	3.8	100	0.38	1.3	96
<b>Lamb</b>							
Shank	11.7	0.49	4.2		0.19	1.6	
Roast shank	12.5	0.51	4.1	98	0.16	1.3	78
Shoulder	15.8	0.62	3.9		0.22	1.4	
Loin	13.0	0.52	4.0		0.17	1.3	
Leg	16.5	0.67	4.1		0.23	1.4	
Roast leg	27.5	1.05	3.8	91	0.33	1.2	84
<b>Pork</b>							
Picnic ham	16.5	0.63	3.8		0.23	1.4	
Butt	12.2	0.48	3.9		0.17	1.4	
Roast butt	22.8	0.89	3.9	100	0.25	1.1	78
Loin	17.4	0.61	3.5		0.24	1.4	
Ham	13.9	0.54	3.9		0.19	1.1	
Roast ham	27.6	1.04	3.8	96	0.30	1.1	78
<b>Beef</b>							
Chuck	17.4	0.68	3.9		0.23	1.3	
Roast chuck	22.7	0.86	3.8	100	0.25	1.1	85
Rib	16.7	0.62	3.7		0.27	1.6	
Loin end	17.6	0.53	3.6		0.23	1.3	
Round	20.6	0.63	3.5		0.27	1.3	
Roast round	33.4	1.27	3.8	110	0.33	1.0	73

TABLE II—*Continued*

Cut of meat	Protein	Phenylalanine			Tryptophan		
		In meat	In protein	Retention	In meat	In protein	Retention
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
<b>Beef organs</b>							
Brain	10.6	0.43	4.1		0.10	1.1	
Brain	10.8	0.43	4.0		0.11	1.0	
Brain	10.3	0.41	4.0		0.12	1.2	
Heart	17.4	0.73	4.2		0.23	1.3	
Heart	17.7	0.74	4.2		0.25	1.4	
Kidney	16.7	0.72	4.3		0.25	1.5	
Kidney	16.4	0.71	4.3		0.25	1.5	
Kidney	16.0	0.69	4.3		0.34	1.6	
Liver	21.0	0.93	4.4		0.34	1.6	
Liver	20.8	0.89	4.3		0.33	1.6	
Liver	20.4	0.87	4.3				
Tongue	20.5	0.78	3.8		0.23	1.1	
Tongue	20.2	0.81	4.0		0.22	1.1	

In Table III are given average values for the tryptophan and phenylalanine content of nine representative types of meat when determined on samples obtained at different periods. The results for lot 1 were obtained by using 1 g. samples and those

TABLE III

*The Tryptophan and Phenylalanine Contents of 2 Lots of Meats*

Kind of meat	Tryptophan		Phenylalanine	
	Lot I	Lot II	Lot I	Lot II
Vcal	1.4*	1.4	3.9	4.0
Lamb	1.3	1.4	4.0	4.0
Pork	1.3	1.4	3.8	3.9
Beef	1.3	1.3	4.2	3.7
Beef brain	1.2	1.1	3.8	4.0
Beef heart	1.3	1.4	4.3	4.2
Beef kidney	1.4	1.5	4.6	4.3
Beef liver	1.6	1.6	4.9	4.3
Beef tongue	1.2	1.1	3.8	3.9

\* Calculated as per cent in the protein (on the basis of 16% N).

for lot 2 by using 10 g. samples. In each case the proper dilutions were made before aliquots were taken for assay. In the first case the assays were set up by hand and the final volume of the assay mixture was 10 ml. In the second case the assays were prepared with a Cannon dispenser and the final assay volume was 2 ml.

## DISCUSSION

The determinations of tryptophan and phenylalanine were made directly on the meat samples. The percentages of these amino acids were calculated on the fresh meat samples directly and also on the basis of 16% N as determined by Kjeldahl method. The percentage of tryptophan in the protein of the muscle tissues was 1.3–1.4%; the amount of tryptophan in brain and in tongue was less, while that in kidney and liver was somewhat higher than this. Beach *et al.* (6) have reported that muscle tissues contain 1.31–1.44% tryptophan, while beef heart contains 1.41; liver, 1.81; kidney, 1.81, and brain, 1.64% (corrected for moisture and ash and calculated to 16% N). These authors used the method of Lugg, modified for use with the spectrophotometer, for these determinations. These same authors have obtained 3.93–4.92% phenylalanine in muscle tissues. The organs contained the following percentages of phenylalanine: beef heart, 5.10; liver, 6.16; kidney, 5.47; and brain, 5.83%. Hier *et al.* (7) have reported 3.9% phenylalanine for beef muscle. The present works show a range of 3.7–4.0% phenylalanine in the muscle tissues. The values obtained for the heart, kidney, and liver are higher than these while the phenylalanine of the other organ samples is comparable to that in the muscle tissues.

It should be pointed out that the results show these values to be reproducible from one lot of meat samples to the next. It should be emphasized that it is important to use fresh samples in which there has been no chance for loss of water or for bacterial decomposition. Likewise, the method is satisfactory when either 2.0 or 10.0 ml. of total assay mixture are employed. Since the meats were well mixed to insure homogeneity and the samples were carefully weighed, the values are just as satisfactory when a 1 g. sample is employed as when 10 g. of the meat were hydrolyzed.

The data on retention indicate a slight loss of tryptophan on cooking. However, denaturation of the protein may have made the tryptophan more difficult to liberate from the protein. Further investigations are needed to clarify this point.



## SUMMARY

1. *Lactobacillus arabinosus* has been used for the direct determination of tryptophan and phenylalanine in fresh and cooked animal tissues and organs.

2. The average tryptophan content of the muscle tissues was 1.3% while the tryptophan content of liver and of kidney was slightly higher, and that of brain and tongue was lower.

3. The average phenylalanine content of all the muscle tissues was 3.9%. Heart, liver, and kidney organs contained more than this, while the tongue and brain contained the same amount of phenylalanine as the muscle tissues.

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# On the Mechanism of Enzyme Action. XXXI. The Mechanism of Methyl-*p*-Methoxycinnamate Formation by *Lentinus lepideus* and Its Significance in Lignification

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## INTRODUCTION

In previous papers of this series experimental evidence was adduced for the course of wood decay observed by the action of certain *Merulii* and *Fomes annosus*. These molds, although deficient in a phosphorylating system, proved to possess the requisite enzymes for the degradation of cellulose, hexoses and pentoses, and dehydrogenating enzymes which act on the alcohol or acetic acid so formed *via* a two-pronged phase sequence to finally give rise to the formation and accumulation of oxalic acid (1). However, the mold *Lentinus lepideus* (Lelep), beside being a perfect alcoholic fermenter, when utilizing glucose or xylose gives rise also to a crystalline compound, *viz.*, methyl-*p*-methoxycinnamate (2). The same product was obtained when the organism acted on white scots pine (3). Inasmuch as molds causing a brown rot preferentially decompose the cellulosic fraction of wood, and in view of the structural relationship between the above mentioned metabolite and known chemical degradation products of lignin, it remained to establish from which fraction of the wood the ester arose. Thus, on the one hand, the degradation of carbohydrates results in methyl-*p*-methoxycinnamate and, on the other hand, this ester has close structural reference to lignin fission products.

\* Communication No. 56 presented before the Sixth International Congress on Cell Research, Stockholm, July, 1947, and the Division of Biological Chemistry at the meeting of the American Chemical Society, New York, 1947. For a preliminary communication, see *Nature* **160**, 224 (1947). These studies were carried out with the aid of grants from the Rockefeller Foundation and the Office of Naval Research.

Two important questions present themselves, therefore, for consideration:

- (1) to determine whether the ester had its origin in the cellulosic or lignin fraction of the wood, and
- (2) to elucidate the mechanism of its formation.

Since the organism preferentially attacks the cellulosic fraction, it would seem that the above compound had its genesis in this entity. The previous finding (2) that *Lelep* produced the ester when grown on glucose would support this view, for *Lelep* when cultivated on cellulose did not give rise to determinable amounts of the ester, due to scanty growth. Its formation from glucose would warrant the assumption that the compound had its origin in the cellulose, since the degradation of the cellulosic fraction of wood by our molds occurs *via* a preliminary hydrolysis.

The observation that *Lelep* causes an alcoholic fermentation of xylose (2), as well as glucose, and that the alcohol so formed was quickly dehydrogenated, indicated that the compound has been synthesized from a common reactive breakdown product arising from both carbohydrates. In addition, resazurin incorporated in the medium was discolored, this observation serving as additional evidence for the presence of a dehydrogenating enzyme system. These findings made it appear that the acetaldehyde formed as a result of the dehydrogenation of ethyl alcohol could be the transient (4) product which then again is enzymatically converted into the ester.

The experiments presented in this paper give conclusive evidence that the C-2 compound which serves as a second switchboard in this multipath enzymatic synthesis is acetaldehyde and constitutes the connecting link in the catabolism of the carbohydrates and the synthesis of a methylated, aromatic compound by *Lelep*.

The medium on which *Lentinus lepideus* was maintained, and the method of isolation and identification of the synthesized product were presented in the preceding report (2). Fully grown mats prepared from glucose medium were used, one mat, after thorough washing with sterile distilled water to remove residual glucose, being transferred to a three liter Fernbach flask containing one liter of medium. Glucose, xylose or glycerol was added (20 g./l.) prior to sterilization. Ethyl alcohol (20 g./l.) was added after sterilization of the medium, employing sterile technique. In intercepting experiments using dimedon to trap acetaldehyde, 0.5 g. of the agent, equivalent to 8.5% of the stoichiometrically required amount, was added to the medium prior to sterilization. This represents the quantity which was tolerated by fully developed mats, without causing measurable detriment to the enzymatic activity of

the mold. The ethyl alcohol formed was quantitatively determined by the method of Janke and Kropacsy (5).

### ISOLATION AND IDENTIFICATION OF ACETALDEHYDE-DIMEDON ADDITION COMPOUND

Two 3-liter Fernbach flasks containing one liter of 1% ethyl alcohol medium supplemented with 0.5 g. of dimedon/l. were inoculated with one fully grown *Lelep* mat, after employing the washing procedure with sterile distilled water. During an incubation period of 30 days, a thin film of platelets was formed on the surface of the inoculated medium. At the end of this period the contents of the flasks were filtered, thoroughly washed with water and oven-dried at 50°C. overnight. The dried mycelium containing the acetaldehyde-dimedon addition compound was ground to a powder and extracted with 200 ml. of ether overnight. Upon evaporating the solvent, 380 mg. of a crude product were obtained. This material was recrystallized from alcohol. Its m.p. was 140°C. (uncorr.). No depression was observed when a mixed melting point with an authentic sample of acetaldehyde-dimedon compound was taken (m.p. 140°C.).

### RESULTS AND DISCUSSION

The results of the formation and disappearance of ethyl alcohol during the dissimulation of glucose and xylose are recorded in Table I.

TABLE I  
*Formation and Dissimulation of Ethanol by the Action of  
Lentinus lepideus on Glucose and Xylose*

Days	Glucose-Ethanol*		Xylose-Ethanol*	
0	206.0	0	246.0	0
5	191.6	3.8	230.0	3.5
6	186.0	9.4	226.0	3.9
7	185.2	13.1	225.2	4.4
8	183.5	15.4	220.1	7.3
9	174.3	16.1	216.0	7.7
10	171.1	11.1	213.3	9.6
11	169.7	9.6	212.0	9.9
13	166.1	7.7	206.1	18.4
15	154.7	7.5	204.0	7.7

\* Results expressed in mg./100 ml.

The dehydrogenation of ethyl alcohol when employed as the sole carbon source is depicted in Fig. 1.

Our findings attest to the formation and subsequent dehydrogenation of ethyl alcohol arising from glucose, xylose and glycerol. The dehydrogenation of ethyl alcohol to acetaldehyde has been substanti-

ated by the isolation and identification of an acetaldehyde-dimedon addition product formed when Lelep was grown on an appropriate medium which was supplemented with dimedon. In a parallel experiment, omitting the trapping agent, appreciable quantities of the ester

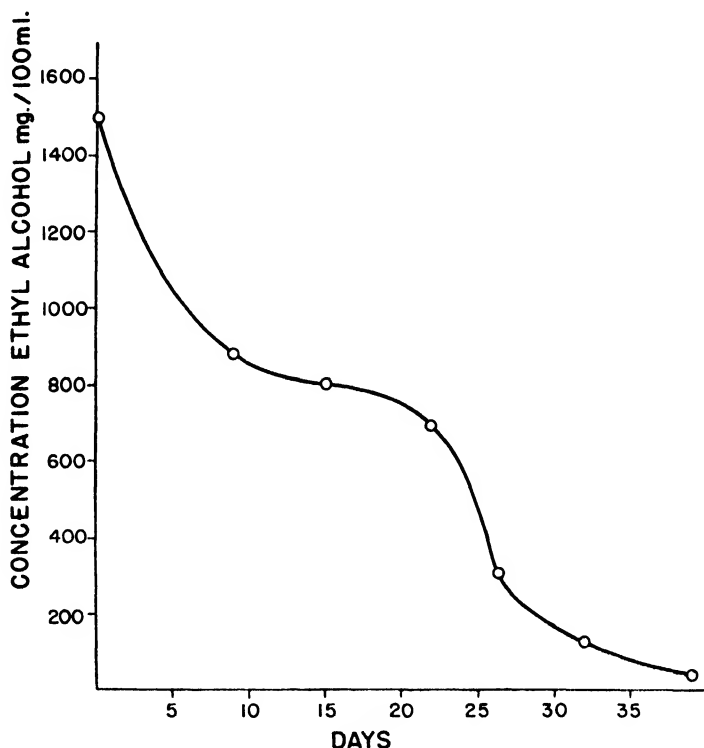


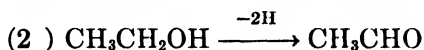
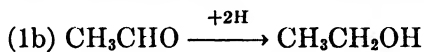
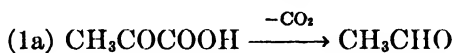
FIG. 1. Dehydrogenation of ethyl alcohol by *Lentinus lepideus*.

were formed from alcohol. The fact that no apparent formation of the crystalline ester took place in the trapping experiment indicated that acetaldehyde is the transient source required to produce the ester. Dimedon, when added to a 2% glucose-containing medium, prevented the formation of the synthetic product, although luxuriant growth took place.

These observations support the view that the acetaldehyde serves as the link between the enzymatic degradation of glucose and/or xylose and the enzymatic synthesis of the ester. Moreover, the acetaldehyde

is involved in 3 enzymatic reactions:

- (1) the decarboxylation of pyruvic acid obtained from the carbohydrates,
- (2) the dehydrogenation of the ethyl alcohol formed, and
- (3) its utilization in the synthesis of the ester:



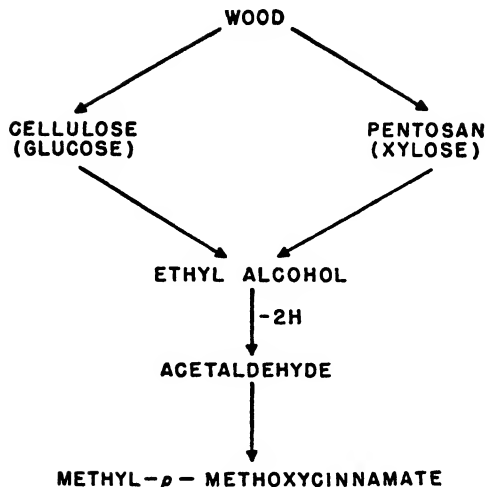
No doubt, the kinetics of reaction (1a) must exceed the rate of reaction (2), and the acetaldehyde is the object of a multifold competition. Moreover, upon allowing the mats to incubate for a long period (2-3 months), it was observed that the crystalline ester formed when the organism was grown on our alcohol medium gradually disappeared. This finding would indicate that certain enzymes, discharged during the slow disintegration of the cells into the medium, utilize the insoluble ester as a carbon source after the alcohol supply has been depleted.

Later, it is intended to study the nature of any additional metabolite formed concomitant with the disappearance of the insoluble material.

### COMMENTS

The conclusions of this investigation answer the query posed at the outset as to whether methyl-*p*-methoxycinnamate had its origin in the cellulosic fraction of wood which is preferentially degraded by Lelep, or in the lignin, degradation products of which possess structures similar to this ester. Having established that the synthetic product arises from the cellulosic fraction, a consideration as to the general scheme of lignification of wood seems to be in order. A picture of the formation of the ester is depicted in Scheme 1.

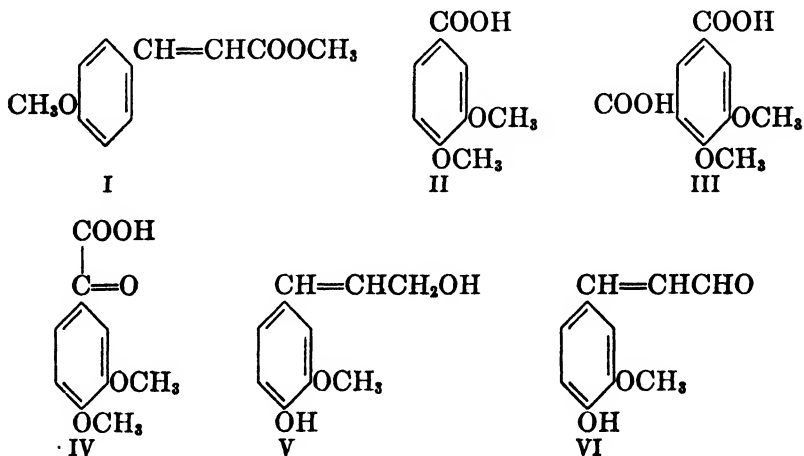
While the origin of lignin is still unknown, speculations continue to appear in the literature as to the possibility of its *direct* formation from carbohydrates. Several (6, 7, 8) suggestions, some of which are of purely meditative origin, have been advanced to explain the nature of



SCHEME 1.

the precursors of lignin and of the mechanism of its formation in the plant.

On the evidence of our findings, the concept of the formation of lignin from the carbohydrates of the wood received experimental support. Methyl-*p*-methoxycinnamate (I) is similar in structure to several fission products of lignin. A few such products which have been obtained from lignin are, veratric acid (II), isohemipinic acid (III), and veratroyl formic acid (IV).



As early as 1897, Klason (9) was of the opinion that lignin is the condensation product of coniferyl alcohol (V). Later (10) he suggested that coniferyl aldehyde (VI) may be the basic building block of lignin.

The similarity between the compounds is apparent. Since methyl-*p*-methoxycinnamate is closely related structurally to the lignin fission products, one could assume that it arose from the lignin portion of the wood. The evidence at hand, however, discloses that the ester is formed *via* a degradation of the cellulose of wood to acetaldehyde, the enzymatic synthesis ensuing from here. This would indicate also that the progress of lignification in woods is governed by the respective rates of the enzymatic carbohydrate degradation and the synthesis of methylated products.

#### SUMMARY

1. The wood-destroying mold, *Lentinus lepideus*, produces methyl-*p*-methoxycinnamate from glucose, xylose, glycerol, and ethyl alcohol.
2. Ethyl alcohol formed from glucose, xylose, and glycerol is dehydrogenated, as indicated by the discoloration of resazurin when incorporated in an alcohol medium.
3. Acetaldehyde, the dehydrogenation product of ethyl alcohol, when trapped with dimedon, does not give rise to the synthesis of the ester which, however, does occur on omitting the trapping agent.
4. Methyl-*p*-methoxycinnamate has its origin in the cellulosic fraction rather than the lignin of wood.
5. The origin of lignin from carbohydrates is discussed.

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## Resistance of Polymyxin to Some Proteolytic Enzymes

Most, if not all, of the antibiotics which have been obtained from spore-forming bacteria have either proven to be polypeptides, *e.g.*, gramicidin (1), tyrocidine (1) and gramicidin S (2), or strongly suspected of being polypeptides, *e.g.*, subtilin (3) and bacillin (4). It was of some interest, therefore, to determine whether proteolytic enzymes had any effect on polymyxin (5), the antibiotic substance formed by *Bacillus polymyxa*. It was found, as described below, that unlike subtilin (3) polymyxin was resistant to inactivation by several crude enzyme preparations. These results, however, do not exclude the possibility of polymyxin having a peptide structure, inasmuch as some naturally occurring polypeptides, *e.g.*, gramicidin, are known to be resistant to proteolytic enzymes (1).

Solutions of polymyxin (500 units/mg.) were prepared in distilled water and adjusted to pH 6 with NaOH. Subtilin<sup>1</sup> was used to check the activity of the various enzymes. It was suspended in distilled water, acidified to pH 2.5 and heated for one hour at 45°C. to induce solution. A 0.1% solution, however, could not be obtained under these conditions. The suspension was readjusted to pH 6.0 and filtered. For our purposes, the resulting solution was considered saturated. The enzymes<sup>2</sup> were prepared as 3% solutions or suspensions in 0.85% NaCl. Both the antibiotic and enzyme solutions or suspensions were clarified and sterilized by filtration through sintered glass. One ml. of active or inactivated (by autoclaving) enzyme solution was added to 1 ml. of the appropriate buffer solution of McIlvaine (6) and this was followed by 1 ml. of either the polymyxin or subtilin solution. The sterile test mixtures were incubated for 22–24 hours at 37°C. Polymyxin was assayed by the agar diffusion method (7) while subtilin was assayed by the agar streak method (8) using *Staphylococcus aureus* H as the test organism.

## RESULTS

Neither pepsin (pH 2.2–4.8), trypsin (pH 4.4–7.5), pancreatin (pH 4.4–7.5), nor erepsin (pH 6.1–7.8), had any effect on polymyxin, whereas subtilin was inactivated to the extent of 75% or more under similar conditions. Typical results are shown in Table I.

<sup>1</sup> Lot 118-T, Western Regional Research Laboratory.

<sup>2</sup> Pepsin (Difco, 1:10,000), pancreatin (Armour), trypsin and erepsin (Wilson).

TABLE I  
*Effect of Crude Pepsin on Polymyxin and Subtilin*

pH of test mixtures	Activity <sup>a</sup> of polymyxin after incubation with		Activity <sup>b</sup> of subtilin after incubation with	
	Pepsin	Inactivated pepsin	Pepsin	Inactivated pepsin
2.2	114	112	1-6	1-48
3.5	112	100	1-12	1-48
4.0	114	102	1-6	1-48
4.8	102	118	<1-6	1-48

<sup>a</sup> Units/ml. Each tube was prepared to contain an estimated concentration of 100 units/ml.

<sup>b</sup> Highest dilution of saturated solution inhibiting growth of *S. aureus*.

It was conceivable that the crude polymyxin used contained impurities which inhibited the enzymes. Therefore, solutions of polymyxin and subtilin were mixed and added to a solution of trypsin or erepsin at pH 7 and incubated. The final concentrations of polymyxin and subtilin were similar to those used in the previous experiments. When assayed for subtilin and polymyxin, again subtilin was found to be inactivated whereas polymyxin was unaffected. It is apparent, therefore, that the resistance of polymyxin, at least to trypsin and erepsin, was not due to inhibition of these enzymes by impurities associated with this antibiotic.

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 July 10, 1947

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## Book Reviews

**Biological Symposia, Vol. XII, 1947.** Edited by W. J. DANN and G. HOWARD SATTERFIELD. 531 pp. Jaques Cattell Press. \$6.50.

Let us begin the review of this volume by saying at once that the book is impressive and the appraisal will be eminently satisfactory. Drs. Dann and Satterfield of two nearby universities, Duke and North Carolina State College, have persuaded the top authorities for each vitamin to contribute chapters—generally in pairs—so that the reader can skip rapidly from a consideration of *in vitro* to *in vivo* methods. Without detailing the long list of distinguished contributors, one finds such inevitable connections as Almquist and vitamin K, Bills and vitamin D, Jukes and pantothenic acid, Mason and vitamin E, and, of course, E. E. Snell for the microbiological determination of nicotinic acid. Vitamin B<sub>6</sub> has merited three articles, a number equalled only by Vitamin A, if we include carotene. Inositol has one chapter—by D. W. Woolley. The newer accessory substances, such as the M-vitamins and folic acid, here referred to as "*Lactobacillus casei* factor," have just crept into the book, in spite of its late date, 1947, which reminds us of our perpetual grouse—the disservice caused by the long time of preparation of symposia volumes and annual reviews.

The book is beautifully produced, the pages being heavy, cream-colored and of absorbent texture, though perhaps not entirely suitable for the laboratory bench-top where it will lie exposed to the inevitable splashings of the assay lab. Won't some enterprising publisher give us a laboratory textbook printed on plastic pages wipeable and impervious to all but the rarest solvents?

In connection with the present work and the laboratory bench, the chapters are uneven in their presentation of working instructions although all show where the instructions may be obtained. One would rather like to see the whole volume rewritten (or a companion volume issued) giving exact working details of currently preferred assay methods. Such details, of course, are given in many cases, on pp. 136-7, for instance, where the care and feeding of rats for riboflavin assay are described minutely, or on pp. 272 *et seq.*, where the estimation of pyridoxin by *Neurospora sitophila* is detailed, and on many other pages too.

We are not equipped to examine authoritatively the chapters on the B-vitamins, especially the microbiological phases. Among the fat-solubles and in the general nutritional picture we are more at home. It is a happy forethought that authors should have been drawn from the Food and Drug Division of the Dept. of Agriculture for human assay (O. L. Kline) and vitamin A methods (Nelson and De Witt), especially with the recent adoption by the U. S. Pharmacopoeia of capsulated Vitamin A acetate as a national standard and the imminent intrusion of synthetic vitamin A into a field previously held by fish oils and extracts. The two chapters on vitamin E by Mason and Harris and by J. G. Baxter are admirable and provide exact working directions. The Mason-Evans-Bacharach technique which counts the live, dead and resorbed

issue of the female white rat gives unusually precise figures with less than the number of rats usual in other assays. Even so, it is being realized that the oxidative condition of the accompanying diet can swing the assays over wide limits so that in the presence of a powerful antioxidant, like amyl hydroquinone, the *mean fertility dose* can be half that needed with a diet compounded with inferior, unprotected fat.

The physicochemical methods reported by Baxter for the various tocopherols are all of recent origin and mark a great advance over the simple Furter-Meyer and Emmerie-Engel methods, yeoman service though these have given. But with new tocopherols or new specific methods being announced almost monthly, we are loath to accept any present procedure as final and remain as encouraged yet unimpressed by the latest "method to supersede all methods" as we were when Cologne was "utterly destroyed" for the 14th, or was it the 41st, time?

These are minor criticisms. The work, as a whole, is the only compilation of its kind, and a place must be made for it on the shelves of the practical nutritionist.

K. C. D. HICKMAN, Rochester, N. Y.

**Recent Progress in Hormone Research. Proceedings of the Laurentian Hormone Conference.** Edited by GREGORY PINCUS. Vol. 1, 1947. 399 pages, 162 figs. Academic Press, Inc., New York, N. Y.

The Laurentian Hormone Conference is a continuation of the Gibson Island Hormone Conference of 1943. This volume contains the papers and discussions of the 1945 meeting. The subject matter is presented in four sections. The section on neuro-humoral relationships contains two papers, one by David Nachmansohn "On the Role of Acetylcholine in the Mechanism of Nerve Activity" and another by Frank A. Beach on "Hormones and Mating Behavior in Vertebrates." Section II, entitled "The Chemistry and Physiology of Adrenal Hormones" is, however, devoted only to adrenal cortex hormones. Papers by E. C. Kendall and T. F. Gallagher, respectively, on the partial synthesis of adrenal cortical steroids contain much important information hitherto unpublished. Some physiological aspects on the suprarenal cortex are well presented by C. N. H. Long and Gregory Pincus. The former discussed "The Relation of Cholesterol and Ascorbic Acid to the Secretion of the Adrenal Cortex," and the latter presented in detail "Studies of the Role of the Adrenal Cortex in the Stress of Human Subjects." Section III is entitled "The Role of Hormones in Metabolic Processes." Leo T. Samuels presented much of his original work on the influence of pituitary hormones, and especially the growth factor, on nutrition and growth in the rat. Charles D. Kochakian, likewise, presented much of his own work on the effects of androgens and related substances on tissues other than accessory sex organs. He concludes that the protein anabolic steroids act in part through the kidney, but that protein catabolic steroids act mainly through the liver. W. U. Gardner, in discussing the action of steroid hormones in experimental carcinogenesis, calls attention to the different types of tumors produced in different strains of mice when treated with estrogenic hormones. He also discusses the genetic and heredity aspects of the tendency of different strains of mice to develop specific types of tumors from estrogen treatment and the action of testosterone propionate in antagonizing the cancerogenic action of the estrogens. The fourth section is devoted to clinical aspects. Ira T. Nathanson covers the "Endocrine Aspects of Human Cancer" with emphasis on the

action of estrogens and androgens and the effects of gonadectomy. Fuller Albright supplied a lecture on "The Effect of Hormones on Osteogenesis in Man." In this paper he presents his evidence for the "N" and "S" hormone actions of adrenal cortical origin in osteogenesis. The latter, "S" or sugar hormone, he reports to inhibit endochondral, endosteal, and probably membranous bone formation. The former, the "N" or nitrogen hormone, is considered to act similarly to testosterone in affecting favorably endochondral and probably endosteal bone formation. The "N" hormone he also considers similar to growth hormone as regards epiphyseal growth. The third lecture in Section IV is by Nathan B. Talbot and Edna H. Sobel on "Certain Factors which Influence the Rate of Growth and the Duration of Growth of Children." The authors' well organized presentation emphasizes the influence of five hormonal factors in statural growth and skeletal maturation. The last lecture by Arthur Grollman is entitled "Experimental Renal Hypertension with Special Reference to its Endocrine Aspects." The author's review indicates that a humoral factor of renal origin is involved in hypertension and that the changes induced in the kidney, in water and sodium metabolism and in elevating blood pressure by certain steroids suggest that other endocrine factors may be involved.

This volume is a very important contribution to endocrinology and future volumes should be equally valuable. The inclusion of the discussions makes the work especially valuable. It is to be hoped that the next annual volumes will become available more promptly after the conference than this one.

F. C. KOCH, Chicago, Ill.

**Injury and Death of Bacteria by Chemical Agents.** By OTTO RAHN. No. 3 of the "Biodynamica Monographs," 183 pp. Biodynamica, Normandy, Missouri, 1945. Price \$3.60.

This book is a reprint of articles previously published in *Biodynamica*. It gives a survey of the basic information concerning the action of chemical disinfectants and antiseptics. Questions such as the selectivity of disinfectants, the influence of temperature, concentration, foreign matter and antidotes are discussed thoroughly, without going into the details of the action of single chemicals. The quantitative relationships and the conclusions which they provide for the understanding of the underlying phenomena are duly considered. The mathematical analysis of the pertinent observations is probably the main contribution of the book. The criticism of the phenol coefficient and the presentation of a new method for the characterization of disinfectants are of practical importance. This new method is based upon the measurement of the length of time in which the standard bacterial suspension is sterilized by various dilutions of the disinfectant.

The influences which produce the antiseptics, the cessation of reproduction are different from those which kill bacteria and are probably not the same in all cases. The most common effect of an antiseptic is the decrease of growth rate, probably caused by the impairment of the synthetic activities of the cells. The special properties of the antiseptic effects of dyes, sulfonamides, weak acids and antibiotics are noted. The sulfonamides differ from other antiseptics in that their effect is not immediate and it becomes apparent only after 3-4 generations of cells. This indicates that they do not act on an essential cell protein but must be incorporated into the cell. The dyes

act by creating an abnormal oxidation-reduction potential and normal multiplication is resumed when the potential is readjusted.

The author believes that the logarithmic death rate of bacteria is of the greatest importance in furnishing an insight into the causes of death. This rate means that the same percentage of all bacteria exposed to certain injury, alive at a given time, will die in the next time unit. The rate corresponds to a mononuclear chemical reaction and indicates that the death of the cells is caused by injury to a single molecule. A large part of the book is occupied by a discussion of the logarithmic death rate, the conclusions which can be drawn from it, and the exceptions which have been observed.

The development of chemotherapy and the use of antibiotics have given great importance to the subject treated in this book. Our present knowledge and its applicability to the actual problems are limited. This critical review of the subject and the indication apparent in the entire book, that mathematical analysis of the observations is necessary, cannot but be helpful to further advance.

LOUIS DIENES, Boston, Mass.

**A Handbook of Commonly Used Drugs.** By MICHEL PIJOAN, Director of the Chemical Foundation Laboratory, University of Colorado, and CLARK H. YAEGER, Chief of the Medical Section, Health and Sanitation Division, Office of Inter-American Affairs, Washington, D. C., Charles C. Thomas, Springfield, Ill. 1947. iv + 198 pp. Price \$3.75.

The book consists essentially of three parts. In the first part groups of drugs are treated according to the physiological systems upon which they act (93 pages). This part concludes with an enumeration of "Antiseptics and Disinfectants" (6 pages) and a discussion of some chemotherapeutic agents (24 pages).

In the second part the "treatment of some common diseases peculiar to the sub-tropical and tropical areas of the western hemisphere" (including helminth infestations) is described (45 pages).

The third part is a collection of miscellaneous lists and tables (18 pages).

In the first part the subject matter is treated in a way customarily used in pharmacology texts. The material is not well balanced and the subdivisions do not facilitate the practical use of the book. For instance, the drugs acting upon the gastro-intestinal tract are represented essentially by a tabulation of cathartic and emetic substances and by a few remarks scattered through the chapter on autonomic drugs. Drugs acting on the eye are not discussed separately and only an incomplete account can be gathered together from various sections.

There is no systematic arrangement of subject matter within the individual chapters under appropriate headings, such as: chemistry, mechanism of action, fate, indications, side effects, ways of administration, dosage. As a result, there is much disorder and repetition. Under many chapter headings divergent matter is found. For instance: sedatives and hypnotics are unjustifiably mixed up. The emetic effect of Apomorphine Hydrochloride is discussed under "Analgesia and Narcosis"; the germicidal and fungicidal action of Iodine forms the introductory paragraph of "Use of Iodine in Thyroid Disease".

The second part uses a clinical approach, grouping the material on the basis of insect vectors. As a result, much material is included which has little bearing on the

use of drugs. With the exception of a few sections, this part is well organized. It is, however, not integrated with the rest of the book.

The main contents of the third part are: five pages of formulae of pharmaceutical preparations, most of which could have been referred to by listing the correct official names; tables of the incubation periods of common infectious diseases, of data on blood, blood chemistry, gastric function and kidney function, and an obstetrical table, none of which would be looked for under the title of this book; a superfluous list of definitions of "terms associated with drugs"; two pages of Latin words and abbreviations, most of them no longer needed for the intelligent writing of prescriptions; deficient samples of prescriptions; and a list of equivalents of weights and measures, which does not adequately take cognizance of the official change from the apothecaries system to the metric system.

The book is not reliable. There are many loose statements which can, at best, be classified as half-truths. The errors are so numerous as to defy an attempt at listing them. Many betray ignorance of basic facts; the majority are indicative of a striking deficiency in knowledge of grammar and of an astonishing degree of carelessness in reading the proofs. Numerous and grave errors are found even in the passages, tables, and structural formulae copied from other texts. A glaring example is the table of the barbituric acid derivatives (page 14), half of which are wrong.

The use of official names and symbols is inadequate, haphazard, and very often incorrect. The only time a specific issue of the U. S. Pharmacopoeia is mentioned (page 62), reference is made to U.S.P. XI.

There are grave omissions. Local Anesthesia is not discussed. Procaine Hydrochloride is not mentioned at all. While Cocaine Hydrochloride is superficially referred to in the text, it does not appear in the index. Opium and Tincture of Opium are not discussed. The ways of administration and the appropriate dosages of Epinephrine Hydrochloride are not adequately stated. The difference in the time course of action between Ergonovine Maleate and Ergotamine Tartarate is not brought out. With the exception of Iodine, antithyroid substances are not mentioned. There is no reference to any anticoagulant but heparin. The pure penicillins are not discussed. Pamaquine Naphthoate is not found amongst the antimalarial agents. The correct chemical name of B.A.L. is not given. This list is far from complete.

The bulk of the book is written in a foggy medical jargon. For example, the "Parasympathomimetic Drugs" are introduced (page 34) as follows:

"In spite of the lack of certain defined mass reactions produced by the parasympathetic system, there are characteristics common to its several distributions. First of all the effects induced by the central nervous system on the visceral organs are mediated concurrently to both sympathetic and parasympathetic apparatuses, and a state of functional integration results. Second, the action of parasympathetic has characteristics, such as protection, conservation and restoration of bodily resources, common to its several divisions."

The introductory paragraphs on pages 19, 22, 45, and 73 are equally torturous to read. However, most of the second part (Chapter XVII) is well written and has few errors of fact and spelling.

At the time of its appearance, 1947, a large part of this book is already out of date. Unreliability makes it useless and—in the hands of uncritical readers—even danger-



ous. The publishers would have done a service to the prospective buyers, to the authors, and to themselves, had they prevented the publication of this book in its present form.

OTTO KRAYER, Boston, Mass.

**X-Ray Diffraction Studies in Biology and Medicine.** By M. SPIEGEL-ADOLF, AND G. HENRY. Grune and Stratton, New York, 1947. vii + 215 pp. Price \$5.50.

When the publication of this book was announced, the reviewer looked forward very eagerly to it, because there was a real need for a critical analysis of the work which has been done by X-ray diffraction on materials of biological interest. The field is an exciting one, and the questions which one tries to answer by any means, such as X-ray diffraction, are pressing ones. Because, however, of the character of the materials, the X-ray diagrams are generally not too detailed, even with the best available techniques. This has led X-ray workers to attempt to squeeze from their results all they could possibly obtain, and often they have squeezed too hard. For this reason particularly, a critical survey of the work was to be looked for. This book, however, does not fulfill these hopes. It is reasonably up to date in the literature quoted, fairly complete and completely uncritical. An undue amount of space, and certainly money, has been spent in the reproduction of many diagrams which to this reviewer's unsophisticated eye look remarkably alike and uninforming. From the point of view of literature references, the bibliography is good, but the book is not the definitive handbook on the subject that the name indicated it might be. If a good bibliography in this field is worth \$5.50, then the reviewer recommends its acquisition.

I. FANKUCHEN, Brooklyn, New York

## AUTHOR INDEX

- A**
- ABBOTT, L. D., JR. Phenolsulfatase activity, 205
- ALBAUM, H. G., AND OGUR, M. An adenine-pentose-pyrophosphate from plant tissues, 158
- ALDERTON, G. See Dimick, 1
- ANANENKO, N. H. See Stansly, 473
- ATKIN, L., AND GRAY, P. P. Fermentation studies with brewers' yeast. I. A synthetic medium, 305
- B**
- BEARD, H. H. See Kopper, 195
- BEILER, J. M. See Martin, 201
- BERRYMAN, G. H. See Grundy, 187
- BOHONOS, N., EMERSON, R. L., WHIFFEN, A. J., NASH, M. P., AND DEBOER, C. A new antibiotic produced by a strain of *Streptomyces lavendulae*, 215
- BRENDEL, R. See Martin, 323
- BREWER, C. R., MICKELSON, M. N., AND WERKMAN, C. H. Effect of growth substrate on the enzymic constitution of *Aerobacter indologenes*, 379
- C**
- CERECEDO, L. R. See Mirone, 324
- D**
- DAVIS, B. D. The estimation of small amounts of fatty acid in the presence of polyoxyethylene sorbitan partial fatty acid esters ("Tween") and of serum proteins, 351
- The preparation and stability of fatty acid-free polyoxyethylene sorbitan monooleate ("Tween" 80), 359
- DAVIS, J. S., JR. See Müller, 39
- DEBOER, C. See Bohonos, 215
- DEUEL, H. J., JR. See Mattson, 65
- See Wiese, 75
- DI CARLO, F. J., AND REDFERN, S.  $\alpha$ -Amylase from *Bacillus subtilis*. I. Purification and physical properties, 333
- $\alpha$ -Amylase from *Bacillus subtilis*. II. Essential groups, 343
- DIMICK, K. P., ALDERTON, G., LEWIS, J. C., LIGHTBODY, H. D., AND FEVOLD, H. L. Purification and some properties of subtilin, 1
- E**
- ELLIS, G. H. See Smith, 81
- ELVEHJEM, C. A. See Greenhut, 459
- See McLaren, 169, 179
- EMERSON, R. L. See Bohonos, 215
- ERRERA, M., AND GREENSTEIN, J. P. Note on the ultraviolet absorption spectra of keto acids and of keto acid peptides, 445
- Effect of various keto acids on the desamidation of glutamine, 449
- ERSHOFF, B. H. Comparative effects of liver and yeast on growth and length of survival of the immature thyroid-fed rat, 365
- ERWAY, W. F. See McShan, 99
- F**
- FEENEY, R. E., LIGHTBODY, H. D., AND GARIBALDI, J. A. Zinc as an essential element for growth and subtilin formation by *Bacillus subtilis*, 13
- FEVOLD, H. L. See Dimick, 1
- FIORE, J. V. See Weiss, 326

- FONTAINE, T. D., MA, R., POOLE, J. B., PORTER, W. L., AND NAGHSKI, J. Isolation of rutin from tomatin concentrates, 89
- FRAPS, G. S. The premature death of white rats fed low levels of carotenes from different sources, 239
- FRAZIER, C. N. See Frieden, 265
- FREED, M. See Grundy, 187
- FRIEDBERG, F. See Winnick, 160
- FRIEDEN, E. H., AND FRAZIER, C. N. The effects of various agents upon the sensitivity of *Staphylococcus aureus* to penicillin, 265
- G**
- GARIBALDI, J. A. See Feeney, 13
- GEIGER, W. B. Interference by streptomycin with a metabolic system of *Escherichia coli*, 227
- GRAY, P. P. See Atkin, 305
- GREENBERG, D. M. See Winnick, 160
- GREENHUT, I. T., POTTER, R. L., AND ELVEHJEM, C. A. The phenylalanine and tryptophan content of meats, 459
- GREENSTEIN, J. P. See Errera, 445, 449
- GROSS, N. H., AND WERKMAN, C. H. Isotopic composition of acetylmethylcarbinol formed by yeast juice, 125
- GRUNDY, W. E., FREED, M., JOHNSON, H. C., HENDERSON, C. R., AND BERRYMAN, G. H. The effect of phthalylsulfathiazole (sulfathalidine) on the excretion of B-vitamins by normal adults, 187
- H**
- HARPER, H. A. The microbiological determination of glutamine in human plasma, 433
- HENDERSON, C. R. See Grundy, 187
- HEPPEL, L. A., PORTERFIELD, V. T., AND PEAKE, E. G. The lipotropic activity of caffeine, theobromine and theophylline, 439
- HOLMAN, R. T. Crystalline lipoxidase. II. Lipoxidase activity, 403
- J**
- JAMESON, E. The determination of plasma and serum protein by electrophoresis. Effect of protein concentration and voltage changes on proportions of different proteins, 389
- JANG, R. See Jansen, 415
- JANSEN, E. F., JANG, R., AND MACDONNELL, L. R. Citrus acetylcholinesterase, 415
- JOHNSON, H. C. See Grundy, 187
- JUNOWICZ-KOCHOLATY, R. See Kocholaty, 55
- K**
- KAVANAGH, F. Chemical determination of pleurotin, an antibacterial substance from *Pleurotus griseus*, 95
- KELLER, D. C. See Medes, 19
- KELLER, E. See McLaren, 169, 179
- KNEEN, E. See Miller, 251
- KNIGHT, C. A., AND OSTER, G. The size of the particles of some strains of tobacco mosaic virus as shown by the electron microscope, 289  
See Oster, 279
- KOCHOLATY, W., AND JUNOWICZ-KOCHOLATY, R. The use of cation exchangers for the concentration and purification of antibodies of basic nature, 55
- KOPPER, P. H., AND BEARD, H. H. Creatinase activity of a strain of *Pseudomonas*, 195
- KURKJIAN, A. See Medes, 19
- L**
- LAUFFER, M. A., AND PRICE, W. C. Electrophoretic purification of southern bean mosaic virus, 115
- LEWIS, J. C. See Dimick, 1
- LIGHTBODY, H. D. See Dimick, 1  
See Feeney, 13
- LUPTON, A. M. See Munro, F. L., 245

## M

- MA, R. See Fontaine, 89
- MACDONNELL, L. R. See Jansen, 415
- McKITTRICK, D. S. The interrelations of choline and methionine in growth and the action of betaine in replacing them, 133
- McLAREN, B. A., KELLER, E., O'DONNELL, D. J., AND ELVEHJEM, C. A. The nutrition of rainbow trout. I. Studies of vitamin requirements, 169  
The nutrition of rainbow trout. II. Further studies with purified rations, 179
- McSHAN, W. H., MEYER, R. K., AND ERWAY, W. F. Effect of estrogens and androgens on the succinoxidase system of rat tissues, 99
- MARTIN, G. J., AND BEILER, J. M. Inhibition of the action of dopa decarboxylase, 201
- MARTIN, G. J., TOLMAN, L., AND BRENDEL, R. The hypotensive action of 7-methylpteroylglutamic acid, 323
- MATTSON, F. H., MEHL, J. W., AND DEUEL, H. J., JR. Studies on carotenoid metabolism. VII. The site of conversion of carotene to vitamin A in the rat, 65
- MEDES, G., KELLER, D. C., AND KURKJIAN, A. Essential fatty acid metabolism. I. Essential fatty acid content of rats on fat-free and pyridoxine-free diets, 19
- MEHL, J. W. See Mattson, 65  
See Wicse, 75
- MEYER, R. K. See McShan, 99
- MICKELSON, M. N. See Brewer, 379
- MILLER, B. S., AND KNEEN, E. The amylase inhibitor of leoti sorghum, 251
- MIRONE, L., AND CERECEDO, L. R. The beneficial effect of xanthopterin on lactation, and of biotin on reproduction and lactation, in mice maintained on highly purified diets, 324

- MÜLLER, O. H., AND DAVIS, J. S., JR. Polarographic studies of proteins and their degradation products. II. Normal values of the "protein index," 39
- MUNRO, F. L., AND LUPTON, A. M. The influence of dicumarol treatment of rabbits, rats and dogs on the thromboplastic activity of their brain suspensions, 245
- MUNRO, F. L., AND MUNRO, M. P. The preparation of prothrombin by adsorption on, and elution from, aluminum hydroxide, 295
- MUNRO, M. P. See Munro, F. L. 295

## N

- NAGHSKI, J. See Fontaine, 89
- NASH, M. P. See Bohonos, 215
- NORD, F. F., AND VITUCCI, J. C. On the mechanism of enzyme action. XXXI. The mechanism of methyl-*p*-methoxycinnamate formation by *Lentinus lepidus* and its significance in lignification, 465  
See Weiss, 326

## O

- O'DONNELL, D. J. See McLaren, 169, 179
- OGUR, M. See Albaum, 158
- OLESON, J. J. See Whitehill, 31
- OSTER, G., KNIGHT, C. A., AND STANLEY, W. M. Electron microscope studies of Dahlem, Rothamsted, and Princeton samples of tobacco mosaic virus, 279  
See Knight, 289

## P

- PEAKE, E. G. See Heppel, 439
- PERLZWEIG, W. A. See Rosen, 111
- POOLE, J. B. See Fontaine, 89
- PORTER, W. L. See Fontaine, 89
- PORTERFIELD, V. T. See Heppel, 439
- POTTER, R. L. See Greenhut, 459
- PRICE, W. C. See LAUFFER, 115

## R

- REDFERN, S. See Di Carlo, 333, 343  
 ROSEN, F., AND PERLZWEIG, W. A. On  
 the "pellagragenic" inactivity of in-  
 dole-3-acetic acid in the rat, 111

## S

- SMITH, S. E., AND ELLIS, G. H. Copper  
 deficiency in rabbits. Achromotrichia,  
 alopecia and dermatosis, 81  
 STANLEY, W. M. See Oster, 279  
 STANSLY, P. G., AND ANANENKO, N. H.  
 Resistance of polymyxin to some  
 proteolytic enzymes, 473  
 SUBBAROW, Y. See Whitehill, 31

## T

- TOLMAN, L. See Martin, 323

## V

- VILLELA, G. G. Pteroylglutamic acid  
 (folic acid) and neoarsphenamine  
 toxicity, 157  
 VITUCCI, J. C. See Nord, 465

## W

- WEISS, S., FIORE, J. V., AND NORD, F. F.  
 On the structure and possible functions  
 of a pigment of *Fusarium Solani* D<sub>2</sub>  
 purple, 326  
 WERKMAN, C. H. See Brewer, 379  
 See Gross, 125  
 WHIFFEN, A. J. See Bohonos, 215  
 WHITEHILL, A. R., OLESON, J. J., AND  
 SUBBAROW, Y. A *Lactobacillus* of  
 cecal origin requiring oleic acid, 31  
 WIESE, C. E., MEHL, J. W., AND DEUEL,  
 H. J., JR. Studies on carotenoid me-  
 tabolism. VIII. The *in vitro* conversion  
 of carotene to vitamin A in the intes-  
 tine of the rat, 75  
 WINNICK, T., FRIEDBERG, F., AND  
 GREENBERG, D. M. Incorporation of  
 C<sup>14</sup>-labeled glycine into intestinal tissue  
 and its inhibition by azide, 160

## SUBJECT INDEX

### A

- Absorption, ultraviolet — spectra of keto acids and of keto acid peptides, ERRERA AND GREENSTEIN, 445
- Acetylsterase, Citrus —, JANSEN, JANG, AND MACDONNELL, 415
- Acetylmethyl carbinol, isotopic composition of — formed by yeast juices, GROSS AND WERKMAN, 125
- Achromotrichia, copper deficiency in rabbits and —, SMITH AND ELLIS, 81
- Acids, see *Keto acids* 6
- Adenine-pentose-pyrophosphate, — from plant tissues, ALBAUM AND OGUR, 158
- Adsorption, see *Prothrombin*
- Aerobacter indologenes, effect of growth substrate on the enzymic constitution of — —, BREWER, MICKELSON, AND WERKMAN, 379
- Alopecia, copper deficiency in rabbits and —, SMITH AND ELLIS, 81
- Aluminum hydroxyde, preparation of prothrombin on, and election from — —, MUNRO AND PLATT MUNRO, 295
- Amylase, — inhibitor of leoti sorghum, MILLER AND KNEEN, 251
- $\alpha$ -Amylase, — from *Bacillus subtilis* (I), purification and physical properties, DI CARLO AND REDFERN, 333; (II), essential groups, DI CARLO AND REDFERN, 343
- Analysis, determination of pleurotin, KAVANAGH, 95; electrophoretic purification of Southern bean mosaic virus, LAUFFER AND PRICE, 115; estimation of small amounts of fatty acids in the presence of polyoxyethylene sorbitan partial fatty acid esters ("tween") and of serum proteins, DAVIS, 351, 359; determination of plasma and serum protein by electrophoresis; the effect of protein concentration and voltage changes on proportions of different proteins, JAMESON, 389; microbiological determination of glutamine in human plasma, HARPER, 433
- Androgens, effect of — on the succinoxidase system of rat tissues, MCSHAN, MEYER, AND ERWAY, 99
- Antagonism, see *Antibiotic*
- Antibacterial (see also *Antibiotic*); pleurotin, — from *Pleurotus griseus*, determination, KAVANAGH, 95
- Antibiotic(s) (see also *Polymyxin*, *Subtilin*, *Tomatin*); use of cation exchangers for the concentration and purification of — of basic nature, KOCHOLATY AND JUNOWICZ-KOCHOLATY, 55; — produced by a strain of *Streptomyces lavendulae*, BOHONOS, EMERSON, WHIFFEN, NASH, AND DEBOER, 215
- Azide, as inhibitor, WINNICK, FRIEDBERG, AND GREENBERG, 160

### B

- Bacillus subtilis* (see also *Subtilin*);  $\alpha$ -amylase from — — (I), purification and physical properties, DI CARLO AND REDFERN, 333; (II), essential groups, DI CARLO AND REDFERN, 343
- Bacteria, see *Aerobacter*, *Antibacterial*, *Antibiotic*, *Bacillus*
- Bases, see *Cation*
- Bean mosaic virus, see *Virus*
- Beer, see *Yeast*
- Betaine, choline, methionine, and — in growth, MCKITTRICK, 133
- Biotin, effect on reproduction and lactation, in mice, MIRONE AND CERECEDO, 324

Blood (see also *Plasma*); hypotensive action of 7-methylpteroylglutamic acid, MARTIN, TOLMAN, AND BRENDDEL, 323

Brain, see *Thromboplastic activity*

## C

Caffeine, lipotropic activity, HEPFEL, PORTERFIELD, AND PEAKE, 439

Carbohydrates, see *Lignification*

Carbon, see *Glycine*

Carotene(s), studies on carotenoid metabolism (VII), site of conversion of — to vitamin A in the rat, MATTSON, MEHL, AND DEUEL, 65; studies on carotenoid metabolism (VIII), in vitro conversion of — to vitamin A in the intestine of the rat, WIESE, MEHL, AND DEUEL, 75; premature death of white rats fed low levels of — from different sources, FRAPS, 239

Cation, use of — exchangers for the concentration and purification of antibiotics of basic nature, KOCHOLATY AND JUNOWICZ-KOCHOLATY, 55

Cecum, a lactobacillus of cecal origin requiring oleic acid, WHITEHILL, OLESON, AND SUBBAROW, 31

Choline, —, methionine, and betaine in growth, MCKITTRICK, 133

Citrus, — acetylsterase, JANSEN, JANG, AND MACDONNELL, 415

Copper, — deficiency in rabbits; achromotrichia, alopecia, dermatosis, SMITH AND ELLIS, 81

Creatinase, — activity of a strain of *Pseudomonas*, KOPPER AND BEARD, 195

## D

DAHLEM . . ., see *Virus*

Death, premature — of white rats fed low levels of carotenes from different sources, FRAPS, 239

Decarboxylase, see *Dopa* . . .

Deficiency, copper — in rabbits; achromotrichia, alopecia, dermatosis, SMITH AND ELLIS, 81

Depressor, see *Inhibition*

Dermatosis, copper deficiency in rabbits and —, SMITH AND ELLIS, 81

Desamidation, see *Glutamine*

Dialysis, see *Enzymes (Amylase)*

Dicumarol, influence of — treatment of rabbits, rats and dogs on the thromboplastic activity of their brain suspensions, MUNRO AND LUPTON, 245

Diet (see also *Carotenes*, *Deficiency*, *Growth*, *Lipotropic*, *Mouse*, *Nutrition*, *Rat*, *Vitamins*); essential fatty acid metabolism (I), MEDES AND KELLER, AND KURKJIAN, 19

Disease, see *Deficiency*, *Pellagra*

Dogs, dicumarol treatment, MUNRO AND LUPTON, 245

Dopa decarboxylase, inhibition of the action of —, MARTIN AND BEILER, 201

## E

Electron microscope, — — studies of various samples of tobacco mosaic virus, OSTER, KNIGHT, AND STANLEY, 279; size of particles of some strains of tobacco mosaic virus as shown by the — —, KNIGHT AND OSTER, 289

Electrophoresis (see also *Virus*); determination of plasma and serum protein by —; effect of protein concentration and voltage changes on proportions of different proteins, JAMESON, 389

Election, see *Prothrombin*

Enzyme(s) (see also *Acetylsterase*, *Amylase*, *Creatinase*, *Dopa decarboxylase*, *Lipoxidase*, *Phenolsulfatase*); effect of growth substrate on the enzymic constitution of *Aerobacter indologenes*, BREWER, MICKELSON, AND WERKMAN, 379; mechanism of — action (XXXI), mechanism of methyl-*p*-methoxycinnamate formation by *Lentinus lepideus*

- and its significance in lignification, NORD AND VITUCCI, 465; resistance of polymyxin to proteolytic —, STANSLY AND ANANENKO, 473
- Escherichia coli*, interference by streptomycin with a metabolic system of —, GEIGER, 227
- Estrogens, effect of — on the succinoxidase system of rat tissues, McSHAN, MEYER, AND ERWAY, 99
- Exchangers, see *Cation*
- Excretion, see *Vitamins*

## F

- Fat (see also *Fusarium Solani*); essential fatty acid metabolism (I), of rats on — free diet, MEDES AND KELLER, AND KURKJIAN, 19
- Fatty acid(s), essential — — metabolism (I), essential — — contents of rats on fat-free and pyridoxine-free diets, MEDES AND KELLER, AND KURKJIAN, 19; estimation of small amounts of — — in the presence of polyoxyethylene sorbitan partial — — esters ("Tween") and of serum proteins, DAVIS, 351; preparation and stability of — — free polyoxyethylene sorbitan monooleate, DAVIS, 359
- Fermentation, — studies with brewers' yeast (I), synthetic medium, ATKIN AND GRAY, 305
- Fish, see *Troul*
- Folic acid, see *Pteroylglutamic acid*
- Fungi, see *Pleurotus griseus*
- Fusarium Solani* D<sub>2</sub> purple, structure and possible functions of a pigment of —, WEISS, FIORE, AND NORD, 326

## G

- Glutamine, microbiological determination of — in human plasma, HARPER, 433; effect of keto acids on the desamidation of —, ERRERA AND GREENSTEIN, 449

- Glycine, incorporation of C<sup>14</sup>-labeled — into intestinal tissue and its inhibition by azide, WINNICK, FRIEDBERG, AND GREENBERG, 160
- Grapefruit, see *Citrus*
- Growth (see also *Lactobacillus*, *Liver*, *Reproduction*, *Yeast*); zinc and — and subtilin formation by *Bacillus subtilis*, FEENEY, LIGHTBODY, AND GARIBALDI, 13; choline, methionine, and betaine in —, MCKITTRICK, 133; effect of — substrate on the enzymic constitution of *Aerobacter indologenes*, BREWER, MICKELSON, AND WERKMAN, 379

## H

- Heavy carbon, see *Acetylmethylcarbinol*, *Glycine*

## I

- Inactivation, see *Enzymes* and *Inhibition*
- Indole-3-acetic acid, pellagragenic activity of — — in the rat, ROSEN AND PERLZWEIG, 111
- Inhibition (see also *Azide*, *Enzymes*, *Penicillin*); of the action of dopa decarboxylase, MARTIN AND BEILER, 201; amylase inhibitor of leoti sorghum, MILLER AND KNEEN, 251; hypotensive action of 7-methylpteroglutamic acid, MARTIN, TOLMAN, AND BRENDDEL, 323
- Intestine (see also *Rat*); C<sup>14</sup>-labeled glycine into intestinal tissue, WINNICK, FRIEDBERG, AND GREENBERG, 160
- Isotopic composition (see also *Glycine*); — — of acetylmethylcarbinol formed by yeast juice, GROSS AND WERKMAN, 125

## K

- Keto acid(s), ultraviolet absorption spectra of — — and of — — peptides, ERRERA AND GREENSTEIN, 445; effect on the desamidation of glutamine, ERRERA AND GREENSTEIN, 449



## L

- Lactation, beneficial effect of xanthopterin on —, in mice, MIRONE AND CERECEDO, 324
- Lactobacillus, a — of cecal origin requiring oleic acid, WHITEHILL, OLESON, AND SUBBAROW, 31
- Lentinus lepidus, mechanism of enzyme action (XXXI), mechanism of methyl-*p*-methoxycinnamate formation by — and its significance in lignification, NORD AND VITUCCI, 465
- Leoti, sorghum see *Sorghum*
- Lignification, mechanism of enzyme action (XXXI), mechanism of methyl-*p*-methoxycinnamate formation by *Lentinus lepidus* and its significance in —, NORD AND VITUCCI, 465
- Lipotropic, — activity of caffeine, theobromine, and theophylline, HEPPEL, PORTERFIELD, AND PEAKE, 439
- Lipoxidase, crystalline — (II), — activity, HOLMAN, 403
- Liver, comparative effects of — and yeast on growth and length of survival of the immature thyroid fed rat, ERSHOFF, 365
- Lycopersicon, see *Tomatin*

## M

- Malnutrition, see *Deficiency*
- Man, microbiological determination of glutamine in human plasma, HARPER, 433
- Meats, Phenylalanine and tryptophan content of —, GREENHUT, POTTER, AND ELVEHJEM, 459
- Metabolism (see also *Carotenes*, *Enzymes*, *Liver*); essential fatty acid — (I), essential acid content of rats on fat-free and pyridoxine-free diets, MEDES AND KELLER, AND KURKJIAN, 19; interference by streptomycin with a metabolic system of *E. coli*, GEIGER, 227
- Methionine, choline, —, and betaine in growth, MCKITTRICK, 133

- p*-Methoxycinnamic acid, see *Methyl-*p*-methoxycinnamate*
- Methylation, see *Choline*
- Methyl-*p*-methoxycinnamate, mechanism of enzyme action (XXXI), mechanism of — formation by *Lentinus lepidus* and its significance in lignification, NORD AND VITUCCI, 465
- 7-Methylpteroylglutamic acid, hypotensive action of —, MARTIN, TOLMAN, AND BRENDEN, 323
- Molds, see *Lentinus lepidus*
- Mosaic virus, see *Virus*
- Mouse, beneficial effect of xanthopterin on lactation, and of biotin on reproduction and lactation, in mice maintained on highly purified diets, MIRONE AND CERECEDO, 324
- Muscle, see *Meats*

## N

- Neoparsphenamine, — toxicity and pteroylglutamic (folic) acid, VILLELA, 157
- Nutrition (see also *Deficiency*, *Growth*, *Meats*); — of rainbow trout (I), studies of vitamin requirements, McLAREN, KELLER, O'DONNELL, AND ELVEHJEM, 169; (II), further studies with purified rations, McLAREN, KELLER, O'DONNELL, AND ELVEHJEM, 179

## O

- Oleic acid (see also *Fatty acids*); a lactobacillus of cecal origin requiring —, WHITEHILL, OLESON, AND SUBBAROW, 31

## P

- Pellagra, pellagragenic inactivity of indole-3-acetic acid in the rat, ROSEN AND PERLZWEIG, 111
- Penicillin, sensitivity of *Staphylococcus aureus* to —, FRIEDEN AND FRAZIER, 265
- Pentahydroxyflavone-rutinoside, see *Rutin*

- Pentose, see *Adenine* . . .
- Peptides, ultraviolet absorption spectra of keto acid —, ERRERA AND GREENSTEIN, 445
- Phenolsulfatase, — activity, DeFORREST ABBOTT, 205
- Phenylalanine, — content of meats, GREENHUT, POTTER, AND ELVEHJEM, 459
- Phosph . . . see *Adenine*
- Phthylsulfathiazole (sulfathalidine), effect on the excretion of B-Vitamins by normal adults, GUNDY, FREED, JOHNSON, HENDERSON, AND BERRYMAN, AND FRIEDEMANN, 187
- Pigment, see *Fusarium Solani*, WEISS, FIORE, AND NORD, 326
- Plant, adenine-pentose-pyrophosphate from — tissues, ALBAUM AND OGUR, 158
- Plasma (see also *Blood*, *Prothrombin*, *Thromboplastic activity*); determination of — and serum protein by electrophoresis; effect of protein concentration and voltage changes on proportions of different proteins, JAMESON, 389; microbiological determination of glutamine in human —, HARPER, 433
- Pleurotin, determination, KAVANAGH, 95
- Pleurotus griseus, determination of pleurotin, antibacterial from —, KAVANAGH, 95
- Polarography, polarographic studies of proteins and their degradation products (II), normal values of the protein index, MUELLER AND DAVIS, 39
- Polymyxin, resistance to proteolytic enzymes, STANSLY AND ANANENKO, 473
- Polyoxyethylene sorbitan, estimation of small amounts of fatty acids in the presence of — — partial fatty acid esters ("Tween") and of serum proteins, DAVIS, 351; preparation and stability of fatty acid-free — — monooleate, DAVIS, 359
- Princeton, see *Virus*
- Protein(s), polarographic studies (II), normal values of the — index, MUELLER AND DAVIS, 39; estimation of serum —, DAVIS, 351; determination of plasma and serum — by electrophoresis; effect of — concentration and voltage changes on proportions of different —, JAMESON, 389
- Proteolytic enzymes, see *Enzymes*
- Prothrombin, preparation of — by adsorption on, and elution from, aluminum hydroxide, MUNRO AND PLATT MUNRO, 295
- Pseudomonas, creatinase activity of a strain of —, KOPPER AND BEARD, 195
- Pteroylglutamic acid (folic acid), — and neoarsphenamine toxicity, VILLELA, 157; hypotensive action of 7-methyl —, MARTIN, TOLMAN, AND BRENDDEL, 323
- Purple, see *Fusarium*
- Pyridoxine, essential fatty acid metabolism (I), of rats on —-free diet, MEDES AND KELLER, AND KURKJIAN, 19
- Pyruvic acid, see *Keto acids*

## R

- Rabbits, copper deficiency in —; achromotrichia, alopecia, dermatosis, SMITH AND ELLIS, 81; dicumarol treatment, MUNRO AND LUPTON, 245
- Radioactive carbon, see *Acetylmethylcarbinol*, *Glycine*
- Rainbow trout, see *Trout*
- Rat(s) (see also *Lipotropic*); essential fatty acid content of — on fat-free and pyridoxine-free diets, MEDES AND KELLER, AND KURKJIAN, 19; carotenoid metabolism (VII), site of conversion of carotene to vitamin A in the —, MATTSON, MEHL, AND DEUEL, 65; carotenoid metabolism (VIII), in vitro conversion of carotene to vitamin A in the intestine of the rat,

- WIESE, MEHL, AND DEUEL, 75; effect of estrogens and androgens on the succinoxidase system of — tissues, McSHAN, MEYER, AND ERWAY, 99; indole-3-acetic acid in the —, pellagra-genic activity, ROSEN AND PERLZWEIG, 111; premature death of white — fed low levels of carotenes from different sources, FRAPS, 239; dicumarol treatment, MUNRO AND LUPTON, 245; comparative effects of liver and yeast on growth and length of survival of the immature thyroid-fed —, ERSHOFF, 365
- Reproduction, effect of biotin on —, in mice, MIRONE AND CERECEDO, 324
- Rothamsted . . . , see *Virus*
- Rutin, isolation from tomatin concentrates, FONTAINE, MA, AND POOLE, AND PORTER AND NAGHSKI, 89
- S
- Salmon . . . , see *Trout*
- Serum, see *Proteins*
- Sex hormones, see *Estrogens*
- Sodium azide, see *Azide*
- Sorbitan, see *Polyoxyethylene* . . . .
- Sorghum, amylase inhibitor of leoti —, MILLER AND KNEEN, 251
- Southern bean mosaic virus, see *Virus*
- Spectrum, ultraviolet absorption spectra of keto acids and of keto acid peptides, ERRERA AND GREENSTEIN, 445
- Staphylococcus aureus, sensitivity to penicillin, FRIEDEN AND FRAZIER, 265
- Streptomyces lavendulae, antibiotic produced by a strain of —, BOHONOS, EMERSON, WHIFFEN, NASH, AND DE-BOER, 215
- Streptomycin, interference by — with a metabolic system of Escherichia coli, GEIGER, 227
- Streptothricin, see *Antibiotics*
- Subtilin, purification and properties, DIMICK, ALDEBERTON, LEWIS, LIGHTBODY, AND FEVOLD, 1; — formation by Bacillus subtilis, zinc essential for, FEENEY, LIGHTBODY, AND GARIBALDI, 13
- Succinoxidase, effect of estrogens and androgens on the — system of rat tissues, McSHAN, MEYER, AND ERWAY, 99
- Sugar, see *Fermentation*
- Sulfathalidine, see *Phthalylsulfathiazole*
- T
- Theobromine, lipotropic activity, HEPPEL, PORTERFIELD, AND PEAKE, 439
- Theophylline, lipotropic activity, HEPPEL, PORTERFIELD, AND PEAKE, 439
- Thromboplastic activity, influence of dicumarol treatment of rabbits, rats and dogs on the — — of their brain suspensions, MUNRO AND LUPTON, 245
- Thyroid, comparative effects of liver and yeast on growth and length of survival of the immature — -fed rat, ERSHOFF, 365
- Tissue(s), see *Intestine, Meats, Plant, Rat*
- Tobacco . . . see *Virus*
- Tomatin, rutin from — concentrates, FONTAINE, MA, AND POOLE, AND PORTER AND NAGHSKI, 89
- Toxicity, see *Neosarsphenamine*
- Trout, nutrition of rainbow — (I), studies of vitamin requirements, McLAREN, KELLER, O'DONNELL, AND ELVEHJEM, 169; (II), further studies with purified rations, McLAREN, KELLER, O'DONNELL AND ELVEHJEM, 179
- Tryptophan, — content of meats, GREENHUT, POTTER, AND ELVEHJEM, 459
- Tween, see *Fatty acids*
- "Tween" 80, see *Polyoxyethylene sorbitan* . . .
- U
- Ultraviolet absorption, see *Spectrum*

## V

Virus, Southern bean mosaic —, electrophoretic purification, LAUFFER AND PRICE, 115; electron microscope studies of Dahlem, Rothamsted, and Princeton studies of samples of tobacco mosaic —, OSTER, KNIGHT, AND STANLEY, 279; tobacco mosaic —, size of particles of strains of — — — as shown in the electron microscope, KNIGHT AND OSTER, 289

Vitamin(s) (see also *Yeast*); nutrition of rainbow trout (I), studies of — requirements, McLAREN, KELLER, O'DONNELL, AND ELVEHJEM, 169; (II), further studies with purified rations, McLAREN, KELLER, O'DONNELL, AND ELVEHJEM, 179

Vitamin A, carotenoid metabolism (VII), site of conversion of carotene to — — in the rat, MATTSO, MEHL, AND DEUEL, 65; carotenoid metabolism (VIII), in vitro conversion of carotene to — — in the intestine of the rat, WIESE, MEHL, AND DEUEL, 75

Vitamin B, effect of phthalylsulfathiazole on the excretion of — by normal adults, GUNDRY, FREED, JOHNSON,

HENDERSON, AND BERRYMAN, AND FRIEDEMANN, 187  
Voltage, see *Proteins*

## W

Wood, see *Lignification*

## X

Xanthopterin, beneficial effect on lactation, and of biotin on reproduction and lactation, in mice, MIRONE AND CERECEDO, 324

## Y

Yeast, isotopic composition of acetylmethylcarbinol formed by — juice, GROSS AND WERKMAN, 125; fermentation studies with brewers' — (I), synthetic medium, ATKIN AND GRAY, 305; comparative effects of liver and — on growth and length of survival of the immature thyroid-fed rat, ERSHOFF, 365

## Z

Zinc, — as an essential element for growth and subtilin formation by *Bacillus subtilis*, FEENEY, LIGHTBODY, AND GARIBALDI, 13

## INDEX OF BOOK REVIEWS

- ANDERSON, A. K., *Essentials of Physiological Chemistry* (WHITE, A.), 164
- ANSON, M. L., AND EDSALL, J. T., *Advances in Protein Chemistry*, Vol. III (NEUBERGER, A.), 165
- DANN, W. J., AND HOWARD, G. (ed.), *Biological Symposia*, Vol. XII (HICKMAN, K. C. D.), 475
- FREDERICQ, P., *Données récentes sur la coagulation du sang* (CHARGAFF, E.), 331
- HAWK, PH. B., OSER, B. L., AND SUMMERSON, W. H., *Practical Physiological Chemistry* (HARROW, B.), 163
- JOSEPHY, E., AND RADT, F., *Elsevier's Encyclopaedia of Organic Chemistry*, Vol. 14, *Tetracyclic and Higher Cyclic Compounds* (PFIFFNER, J. J.), 330
- KONIGSBERGER, V. J., SLIJPER, E. J., AND VONK, H. J. (ed.), *Tabulae Biologicae*. Vol. XXI. *Digestion*. Part 1. Part 1 ed. by VONK, H. J., MANSOUR-BEK, J. J., AND SLIJPER, E. J. (SCHEER, B. T.), 162
- PIJOAN, M., AND YAEGER, C. H., *A Handbook of Commonly Used Drugs* (KRAYER, O.), 478
- PINCUS, G. (ed.), *Recent Progress in Hormone Research. Proceedings of the Laurentian Hormone Conference* (KOCH, F. C.), 476
- RAHN, O., *Injury and Death of Bacteria by Chemical Agents* (DIENES, L.), 477
- SPIEGEL-ADOLF, M., AND HENRY, G., *X-Ray Diffraction Studies in Biology and Medicine* (FANKUCHEN, I.), 480
- SUMNER, J. B., AND SOMERS, G. F., *Chemistry and Methods of Enzymes* (SCHLENK, F.), 331
- SZENT-GYÖRGYI, A., *Chemistry of Muscular Contraction* (MEYERHOF, O.), 166
- WAKSMAN, S. A., *Microbiological Antagonisms and Antibiotic Substances* (2nd ed.) (KAVANAGH, F.), 329

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VOLUME 16



1948

ACADEMIC PRESS INC. PUBLISHERS  
NEW YORK, N. Y.



## CONTENTS OF VOLUME 16

No. 1, JANUARY, 1948

JOSE M. GONÇALVES AND JESSE P. GREENSTEIN. Enzymatic Hydrolysis of $\alpha,\alpha$ -Di(glycylamino) propionic Acid.....	1
BABETTE TAYLOR, JESSE P. GREENSTEIN AND ALEXANDER HOLLAENDER. Effects of X-Radiation on Sodium Thymus Nucleate.....	19
ANNABEL AVERY AND F. L. MUNRO. Chemical and Electrophoretic Studies of Fibrinogen Prepared by Various Methods	33
W. V. CRUESS AND JAS. SUGIHARA. The Oxidase of the Olive...	39
HAROLD D. WEST AND RAVEN RIVERA ELLIOTT. The Role of Pantothenate in Sulfapyridine-Induced Achromotrichia.....	47
ALLAN H. BROWN AND JAMES FRANCK. On the Participation of Carbon Dioxide in the Photosynthetic Activity of Illuminated Chloroplast Suspensions.....	55
JAQUES BOURDILLON. Crystalline Pepsin-Resistant Protein from Skeletal Muscle.....	61
B. F. CHOW, R. D. SEELEY, J. B. ALLISON AND W. H. COLE. The Effect of Repletion on the Plasma Proteins in the Dog Measured by Electrophoretic Analysis.....	69
D. PERLMAN. Desthiobiotin and O-Heterobiotin as Growth Factors for "Normal" and "Degenerate" Strains of <i>Clostridia</i>	79
R. H. BROH-KAHN, I. ARTHUR MIRSKY, GLADYS PERISUTTI AND JEAN BRAND. The Hexosemonophosphatase System (Glucose-6-Phosphatase) of the Liver.....	87
DONALD E. BOWMAN. Further Differentiation of Bean Trypsin-Inhibiting Factors.....	109
LOIS M. ZUCKER, THEODORE F. ZUCKER, VIRGINIA BABCOCK AND PATRICIA HOLLISTER. Zoopherin: Nutritional Factor for Rats Associated with Animal Protein Sources.....	115
ROLLIN D. HOTCHKISS. A Microchemical Reaction Resulting in the Staining of Polysaccharide Structures in Fixed Tissue Preparations.....	131
FREDERICK T. WOLF. The Amino Acid Metabolism of <i>Penicillium chrysogenum</i> Q-176.....	143
LETTERS TO THE EDITORS:	
ARTTURI I. VIRTANEN AND ANTTI LOUHIVUORI. Estimation of L-Aspartic Acid with Aspartase.....	151
BOOK REVIEWS.....	153



JOSEPH V. FIORE. On the Mechanism of Enzyme Action. XXXII. Fat and Sterol in <i>Fusarium lini</i> Bolley, <i>F. lycopersici</i> , and <i>F. solani</i> D <sub>2</sub> Purple. . . . .	161
ARTTURI I. VIRTANEN AND J. DE LEY. The Enzyme Activity and Nitrogen Content of Bacterial Cells. . . . .	171
FRANCISCO GIRAL AND MARIA LUISA CASCAJARES. Mexican Turtle Oils. I. . . . .	179
JOSÉ GIRAL, FRANCISCO GIRAL AND MARIA LUISA GIRAL. Mexican Turtle Oils. II. <i>Chelone mydas</i> , Linn. . . . .	183
FRANCISCO GIRAL AND ANDRES MARQUEZ. Mexican Turtle Oils. III. <i>Caretta caretta</i> , Linn. . . . .	189
FRANCISCO GIRAL. Mexican Turtle Oils. IV. <i>Lepidochelis olivacea</i> Esch. . . . .	193
HELEN Y. ZIMMERBERG. The Metabolism of Various Phthalein Dyes by Rat Liver Slices. . . . .	197
CLARY J. FISCHER AND ROY O. GREEP. Studies on Activation of Purified Alkaline Phosphatase. . . . .	201
J. A. BAIN AND H. F. DEUTSCH. Studies on Lactoglobulins. . . . .	223
TSOO E. KING AND VERNON H. CHELDELIN. Pantothenic Acid Studies. II. The Influence of Glutamic Acid and Pantoic Acid on Yeast Growth. . . . .	233
EUGENE ROBERTS AND CHRISTOPHER CARRUTHERS. Adenylpyrophosphatase Activity in Epidermal Carcinogenesis in Mice. . . . .	241
JOHN P. BOWLAND, MARION E. ENSMINGER AND TONY J. CUNHA. Need for an Unidentified Factor or Factors for Growth, Reproduction, and Lactation of Rats Fed Purified Rations. . . . .	257
WILLIAM D. ROSENTHAL. Fatty Acid Transformations by Anaerobic Bacteria. . . . .	263
ERNESTO SODI PALLARES AND HECTOR MARTINEZ GARZA. The Structure of the Alkaloid of Yolochochitl. . . . .	275
M. F. MALLETT, S. LEWIS, S. R. AMES, J. M. NELSON AND C. R. DAWSON. The Preparation of Mushroom Tyrosinase. . . . .	283
P. S. KRISHNAN. Oxygen Absorption Method for the Estimation of Coenzyme I. . . . .	291
FORREST G. HOUSTON AND R. C. BURRELL. The Isolation of Inulin and an Unidentified C <sub>20</sub> Compound from the Roots of <i>Solidago canadensis</i> L. . . . .	299
REGINALD A. SHIPLEY, ETHEL BUCHWALD CHUDZIK AND PAUL GYÖRGY. The Effect of Extirpation of Various Endocrine Glands on the Production of Fatty Liver. . . . .	301
LETTERS TO THE EDITORS:	
A. Z. HODSON. Inhibitory Effect of Pure and Semipurified Proteins on the Activity of Hog Kidney Conjugase. . . . .	309

HANS LINEWEAVER, A. A. KLOSE AND GORDON ALDERTON. An Alternate Step for the Isolation of Subtilin.....	311
JEAN MAYER AND W. A. KREHL. Scorbatic Symptoms in Vitamin A-Deficient Rats.....	313
BOOK REVIEWS.....	315

# No. 3, MARCH, 1948

MAX A. LAUFFER, H. L. CARNELLY AND E. MACDONALD. Ther- mal Destruction of Influenza A Virus Infectivity.....	321
RICHARD E. ROSENFELD AND HAROLD S. TUFT. Preliminary Observations on the Adsorption of Prothrombin by Barium Sulfate.....	329
HARRY G. ALBAUM AND MILTON KLETZKIN. Adenosine Triphos- phate from <i>Drosophila melanogaster</i> .....	333
R. BRUCE MERRIFIELD AND MAX S. DUNN. Microbiological Determination of Cytosine, Uracil and Thymine.....	339
H. L. MITCHELL, W. G. SCHRENK AND H. H. KING. Effect of High-Moisture Storage on Preservation of Carotene in De- hydrated Alfalfa Meal.....	343
DYSON ROSE. The Amylase of <i>Bacillus polymyxa</i> .....	349
ROBERT JOHN EVANS. Sulfur Amino Acid Nutrition of Some Lactic Acid-Producing Bacteria.....	357
A. I. LANSING, T. B. ROSENTHAL AND M. H. AU. Ultrafilterable and Non-Ultrafilterable Calcium in Normal, Hyperplastic Epidermis and Squamous Cell Carcinoma.....	361
MARIAN E. SWENDSEID, R. A. BROWN, O. D. BIRD AND R. A. HEINRICH. Comparative Study of Pteroylglutamic Acid and Its Hexaglutamyl Conjugate in the Correction of Sulfasuxi- dine-Induced Leucopenia in the Rat. Effect of Conjugase Inhibitor.....	367
JOHN R. TOBIN, JR., DELBERT BERGENSTAHL AND C. HAROLD STEFFEE. The Relationship of Protein Reserves to the Pro- duction of Hyaluronidase and Antihyaluronidase.....	373
W. H. MCSHAN, W. F. ERWAY AND ROLAND K. MEYER. Malic Dehydrogenase and Cytochrome Oxidase of Lutein and Other Ovarian Tissues During Pregnancy and Lactation.....	379
H. H. THORNBERRY AND H. W. ANDERSON. Synthetic Medium for <i>Streptomyces griseus</i> and the Production of Streptomycin	389
ROBERTA MA AND THOMAS D. FONTAINE. <i>In Vitro</i> Antibiotic Activity of Crystalline Tomatine Toward <i>Candida Albicans</i> . Antagonistic Effect of Rutin and Quercetin.....	399
LEW CUNNINGHAM, JOAN M. BARNES AND W. R. TODD. Main- tenance of Carbohydrate Stores before and after Insulin Ad- ministration in Rats Prefed Diets Containing Added Glycine	403
ZACHARIAS DISCHE. A New Specific Color Reaction of Galactu- ronic Acid.....	409

C. C. CLAYTON AND C. A. BAUMANN. Effect of Fat and Calories on the Resistance of Mice to 2,4-Dinitrotoluene.....	415
WALTON B. GEIGER. Antibacterial Unsaturated Ketones and Their Mode of Action.....	423
W. A. ROBBIE AND P. J. LEINFELDER. Cyanide Inhibition of Retinal Respiration in Bicarbonate Buffer.....	437
HANS LINEWEAVER, HERMAN J. MORRIS, LEO KLINE AND ROSS S. BEAN. Enzymes of Fresh Hen Eggs.....	443
LETTERS TO THE EDITOR:	
BENTON NISMAN, MARCEL RAYNAUD AND GEORGES N. COHEN. Extension of the Stickland Reaction to Several Bacterial Species.....	473
P. S. KRISHNAN. The Preparation of Apyrase from Potato.	474
BOOK REVIEWS.....	477
AUTHOR INDEX.....	481
SUBJECT INDEX.....	485
INDEX OF BOOK REVIEWS.....	492

# Enzymatic Hydrolysis of $\alpha,\alpha$ -Di(glycylamino)propionic Acid

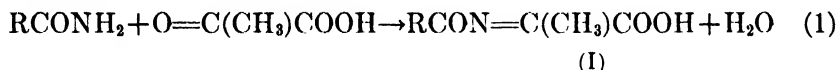
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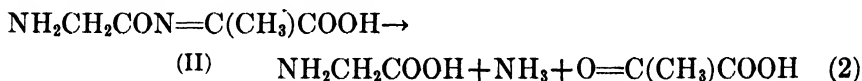
Received July 31, 1947

## INTRODUCTION

The dehydropeptides of  $\alpha$ -aminoacrylic acid (dehydroalanine (I)) may be considered essentially as a combination of one mole of pyruvic acid with one mole of the corresponding acid amide with the elimination of the elements of water (1-3):



When R contains an  $\alpha$ -amino acyl group, as in glycyldehydroalanine (II), the dehydropeptide is susceptible to the action of an enzyme which is widespread and highly active in all animal and plant tissues studied, and which splits the substrate into products which include pyruvic acid and ammonia in equimolar proportions, and to which the designation of dehydropeptidase I has been applied (4-7).

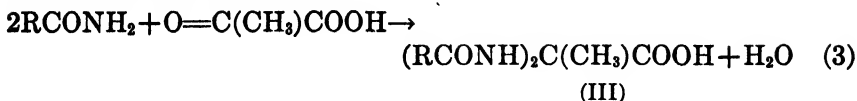


The possibility has been pointed out that the dehydropeptides may actually be formed in tissues by a condensation between  $\alpha$ -keto acids and amino acid amides (8), and the results of experiments on the effect of pyruvate on the rate of desamidation of such naturally occurring amides as glutamine and asparagine have been interpreted on this basis (9-14).

Pyruvic acid, however, can condense not only with one but also with two moles of amino acid amides, forming  $\alpha,\alpha$ -di(acylamino)propionic

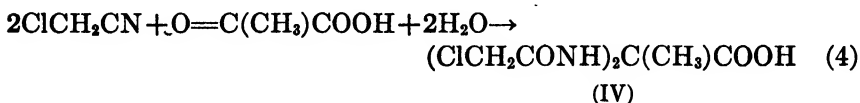
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acid peptides (1) (III).

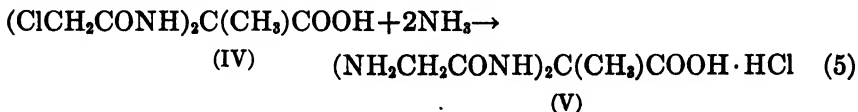


The  $\alpha,\alpha$ -di(acetyl) and  $\alpha,\alpha$ -di(chloroacetyl) derivatives of this class of compounds were described by Bergmann and Grafe (1). Di(chloroacetylamino)propionic acid was obtained as a small byproduct of the synthesis of chloroacetyldehydroalanine through the condensation of pyruvic acid with chloroacetamide. Di(acetamino)propionic acid was formed by the condensation of an excess of acetamide with pyruvic acid; on heating with glacial acetic acid, one mole of acetamide is split off, and acetyldehydroalanine can be readily isolated.

The relation between dehydroalanyl peptides and  $\alpha,\alpha$ -di(acylamino)-propionic acid peptides makes it desirable to acquire information on the possible biologic role of the latter class of substances. Prior to such a study, however, improved methods of preparing such compounds have had to be developed. Reproducibly high yields of  $\alpha,\alpha$ -di(acetamino)propionic acid by the method of condensing pyruvic acid with acetonitrile in chilled concentrated sulfuric acid have been reported (9), and this method has been successfully extended to the synthesis of  $\alpha,\alpha$ -di(chloroacetamino)propionic acid (IV):



$\alpha,\alpha$ -Di(glycylamino)propionic acid monohydrochloride (V) was prepared from the corresponding di(chloro)-derivative by amination with aqueous ammonia, and its characterization and susceptibility to enzymatic attack forms the basis of this report.



#### EXPERIMENTAL PROCEDURE

##### *$\alpha,\alpha$ -Di(chloroacetamino)propionic Acid (IV)*

Sixty g. of freshly distilled pyruvic acid was dissolved in 500 cc. of chilled, concentrated sulfuric acid. The solution was placed in an ice-salt bath, and 100 g. of re-

distilled chloroacetonitrile was added slowly with stirring. The temperature was never allowed to rise above 15°C. The addition took about 2 hours to complete, at the end of which time the solution was highly viscous. It was poured in a thin stream over 4 l. of ice shavings with vigorous stirring. A white precipitate appeared almost immediately. After the ice had melted, the precipitate was filtered by suction, washed several times with ice water, and crystallized twice from hot water. The product appeared as large, glistening prisms, in 33% yield, based on the amount of chloroacetonitrile employed. The melting point was 201°C. Bergmann and Grafe (1) reported a melting point of 199°C. for their product which had been crystallized from methanol. Nitrogen found was 10.8%; calculated, 10.9%.

*$\alpha,\alpha$ -Di(glycylamino)propionic Acid  
Monohydrochloride (V)*

Twenty g. of di(chloroacetamino)propionic acid was dissolved in 200 cc. of ammonia water at 0.9 specific gravity (28%) and the solution kept at 37°C. for 36 hours. The solution was then evaporated *in vacuo* at 35°C. to a colorless syrup. The residue was dissolved in 50 cc. of water, and the solution treated with 200 cc. of absolute alcohol. The peptide precipitated as an oil which rapidly hardened. Three repetitions of this procedure yielded a white precipitate of the peptide hydrochloride, which was filtered, washed successively with alcohol and ether, and finally dried over  $P_2O_5$ . The yield was 16 g., or 80% of the theory. The melting point was 199°C. with decomposition. The peptide is very soluble in water, insoluble in non-aqueous solvents.

The product is slightly hygroscopic and must be carefully dried for analysis. It contains a small amount of impurity which repeated precipitation from alcohol-water mixtures was unable to remove. To characterize the compound adequately, its elemental analysis, electrometric titration curve, and hydrolytic products were determined. The results of the enzyme studies at maximal splitting of the compound also assist in establishing its essential composition.

Calculated C 33.0, H 5.9, N 22.0, Cl (ionic) 13.9

Found C 33.9, H 6.2, N 20.9, Cl (ionic) 12.7

2.55 grams of di(glycylamino)propionic acid monohydrochloride were dissolved in 95 cc. of 2 N HCl and the solution boiled under a reflux condenser for 2 hours. The solution was brought to exactly 100 cc., and 1 cc. aliquots were used for determinations of free ammonia and of pyruvic acid.

Each cc. of the hydrolyzate yielded 2.7–2.8 mg. of free ammonia, or 96–100% of the theoretical for 2 atoms of hydrolyzable ammonia nitrogen per mole of di(glycylamino)propionic acid HCl. Essentially similar findings were noted with hydrolyzates of di(chloroacetamino)propionic acid and of di(acetamino)propionic acid. The two nitrogen atoms are those linked to the tertiary carbon atom in the compounds.

Each cc. of the hydrolyzate yielded 8.5–8.6 mg. of pyruvic acid, or 96–98% of the theoretical for 1 mole of pyruvic acid per mole of peptide. The pyruvic acid was determined spectrophotometrically as the 2, 4-dinitrophenylhydrazone from its absorption curve between 4000 and 5000A and its molar extinction coefficient at the 4400A peak (*cf.* 22).

The remainder of the hydrolyzate of di(glycylamino)propionic acid HCl, 90 cc., was made alkaline to phenolphthalein with 5 *N* NaOH, and was evaporated to 20 cc. *in vacuo* to remove the ammonia. The condensate was chilled, filtered from sodium chloride, and treated with 3.5 g. of freshly-distilled benzoyl chloride. After shaking the mixture for 40 minutes at 5°C. on the machine, chilled 5 *N* HCl was added to congo blue reaction. The yield of dried, crude hippuric acid was 2.6 g. or 80% of the theoretical for 2 moles of glycine per mole of peptide. After recrystallization from water, the product weighed 2.2 g., melted at 186°C., and yielded 7.6% nitrogen, theory 7.8%.

The nature of the small amount of impurity, estimated at about 3-5%, in the preparation of di(glycylamino)propionic acid HCl is not known, but, from the frequent type of impurity encountered when aminating  $\alpha$ -halogenated fatty acid derivatives, and from the impossibility of separating it from alcohol-water mixtures, it is believed to be  $\alpha$ -glycylamino- $\alpha$ -hydroxyacetamino-propionic acid. Two other preparations of di(glycylamino)propionic acid HCl yielded practically similar results and possessed nearly identical properties with the preparation described.

The  $\alpha,\alpha$ -di(acetamino)propionic acid has been described (1, 9).  $\alpha$ -Acetamino- $\alpha$ -aminopropionic acid was prepared by the method of Bergmann and Grafe (1).

Chloroacetyl-DL-alanine was prepared by a modification of the method of Fischer (15), whereby the mixture following acidification was not evaporated to dryness but was shaken out several times with ethyl acetate, the ethyl acetate extracts being combined, dried, and evaporated to a low bulk *in vacuo*. The peptide crystallized out on chilling the solution and, after drying, melted at 127°-128°C. The melting point given by Fischer was 126°-127°C. The yield of the product was two-thirds of that given by Fischer; but the ease of operation is greater, and the time required is shorter. Glycyl-DL-alanine was prepared from the chloroacetyl derivative in the usual manner (15).

Glycine amide hydrochloride was prepared by the method of Bergell and Wülfing (16), which involves the careful amination of chloroacetamide with chilled aqueous ammonia. It was found necessary to follow the directions of Bergell and Wülfing rather closely. Attempts to prepare glycine amide by adding chloroacetylchloride dropwise to chilled concentrated aqueous ammonia, or by shaking ethylbromoacetate with chilled concentrated aqueous ammonia, led in each case to the formation of the hydrochloride or the hydrobromide of iminodiacetamide. Since the melting points of the hydrochloride of glycine amide (186°C.) and of iminodiacetamide (234°C.) are sufficiently separated, the compounds can be readily distinguished.

### *Enzymatic Techniques*

The tissues studied were freshly removed from decapitated rats, ground in a glass mortar with sand, and extracted with 3 times the volume of distilled water. After light centrifugation, the supernatant was used as the source of the enzyme. The digests were prepared by mixing 1 cc. of the tissue extract with 2 cc. of 0.15 *M* borate buffer at the pH desired, and 1 cc. of either water or 25 micromoles of substrate. All data were corrected for the extract blanks.

After incubation of the digests, ammonia nitrogen was determined by aeration into acid traps with subsequent nesslerization according to the method described (5) Pyruvic acid was determined by the method described (17).

### *Pyruvic Acid and Ammonia Recovery Experiments*

Tissues contain enzymes which oxidize or utilize pyruvic acid for many purposes, and the pyruvic acid determined in tissue digests may be too low owing to these factors. Recovery experiments performed by adding known amounts of sodium pyruvate to tissue extracts under conditions identical with those used for the digestion of the substrates revealed that, in dialyzed extracts of kidney, 90–95% of the added pyruvate could be recovered after 4 hours of incubation. With dialyzed extracts of liver nearly 100% of the added pyruvate could be recovered. When fresh tissue extracts were used, the recovery of pyruvic acid was somewhat lower, of the order of 80% of the added material. In all cases, added ammonia was recovered from 95–100% after 4 hours of incubation.

All substrate solutions except one were entirely stable on standing for several weeks in the ice chest and showed no evidence of the presence of either ammonia or pyruvic acid. The exception was  $\alpha$ -acetamino- $\alpha$ -aminopropionic acid, which broke down rapidly in solution to yield equimolar quantities of ammonia and pyruvic acid (see below).

### *Absorption Curve of Di(glycylamino)propionic Acid Monohydrochloride in the Ultraviolet*

Glycyldehydroalanine, by virtue of its capacity for tautomerism, possesses a characteristic absorption in the ultraviolet region of the spectrum, with a band at 2400A (6). Other dehydropeptides have a similar absorption (7). Di(glycylamino)propionic acid may be considered to be glycyldehydroalanine in which the double bond has been saturated by the substitution of a molecule of glycine amide. The absorption characteristics of the dehydropeptide are no longer present, and the spectrum of di(glycylamino)propionic acid reveals only a general absorption in the ultraviolet (Fig. 1).

### *Electrometric Titration Curve of Di(glycylamino)propionic Acid Hydrochloride<sup>2</sup>*

The titration at 25°C. of a 0.02 *M* solution of di(glycylamino)-propionic acid in water was performed by adding 1 *N* sodium hydroxide or 1 *N* hydrochloric acid through a microburette and by noting the pH of the mixture after each addition. The method of drawing the titration curve and of calculating the dissociation constants has been described (18). In Fig. 2, the points are those experimentally determined; the curve is theoretical and is based upon the following constants:  $pK_1 = 1.8$ ,  $pK_2 = 8.1$ ,  $pK_3 = 8.1$ . No correction was made for the small

<sup>2</sup> Titration performed by Drs. Errera and Shack.



amount of impurity ( $< 5\%$ ) in the compound, and the slight deviations in the theoretical curve from the experimental points may be attributed to this factor. The constants calculated may be in error by as much as 0.1 pK unit. However, the close concordance between the curve relating

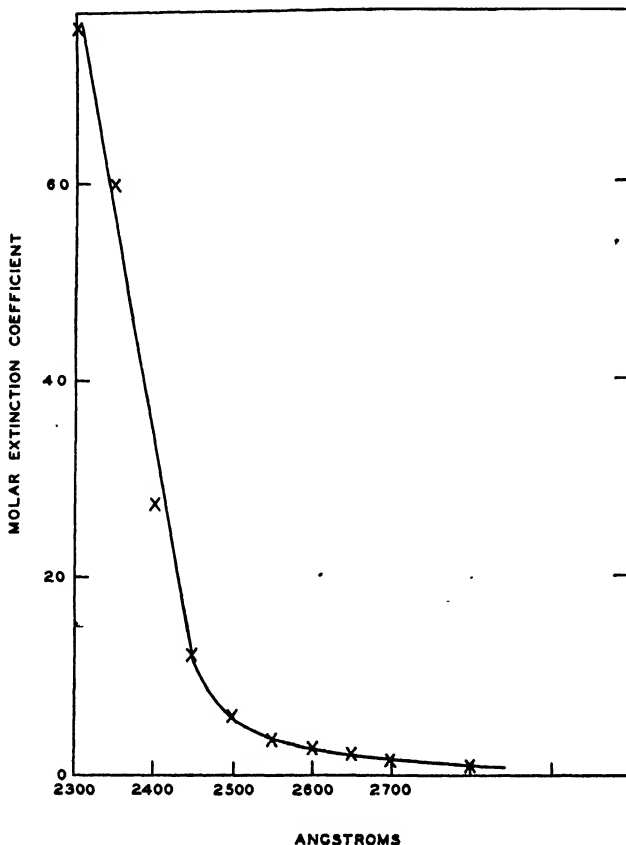


FIG. 1. Ultraviolet absorption spectrum of  $\alpha, \alpha$ -di(glycylamino)propionic acid HCl at 0.025  $M$  concentration in water.

to 3 ionizable groups and the experimental data establishes and confirms the structure of the compound. Di(glycylamino)propionic acid should have two amino groups and one carboxyl group. The acid  $pK_1$  of 1.8 relates to the dissociation of the carboxyl group and is more acid than the carboxyl  $pK$  of dipeptides (about 3.2) in general. This is probably due to the fact that di(glycylamino)propionic acid possesses

two peptide bonds, and this linkage is known to have the effect of increasing the dissociation of carboxyl groups (19). The alkaline  $pK_2$  and  $pK_3$  values of 8.1 relate to the dissociation of the two free amino groups, and it is interesting to note that the two groups dissociate apparently independently of each other. There is no apparent influence of one amino group on the dissociation of the other, such as there is in lysine (18). and it may be that the glycylamino residues in the compound are either so far separated from each other as to be mutually

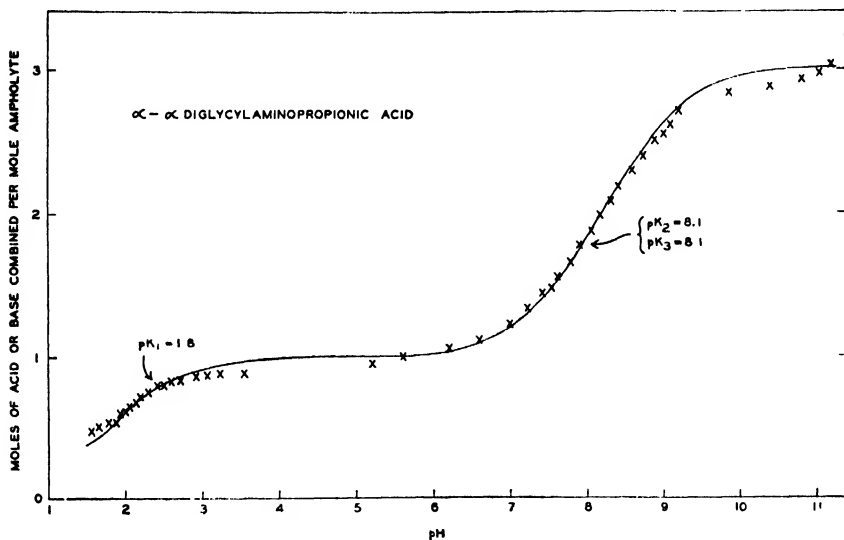


FIG. 2. Electrometric titration curve of  $\alpha,\alpha$ -di(glycylamino)propionic acid hydrochloride at 25°C. The points are experimental. The curves are theoretical, and are based upon the following constants:  $pK_1 = 1.8$ ,  $pK_2 = 8.1$ ,  $pK_3 = 8.1$ .

independent, or else, as in cystine peptides (20), the duplicating groups behave as if they belonged to separate molecules in solution. The amino groups of the diglycylamino residues possess  $pK$  values not far from that of glycine amide which is 7.9 or of glycylalanine which is 8.3 (19).

*Ammonia and Pyruvic Acid Produced Enzymatically  
from Di(glycylamino)propionic Acid*

To see whether the di(acylamino)propionic acids are susceptible to enzymatic hydrolysis and whether ammonia and pyruvic acid are

produced thereby, di(glycylamino)propionic acid hydrochloride, di(chloroacetamino)propionic acid, and di(acetamino)propionic acid were each incubated with aqueous extracts of various fresh rat tissues for 4 hours (pH 8.0) at 37°C. The last two compounds were brought into aqueous solution at 0.025 *M* by the addition of enough 1 *N* sodium hydroxide to neutralize the free carboxyl group. The di(glycyl) peptide was readily soluble in water and gave a solution only faintly acid to litmus. At the end of the incubation period, no increase in ammonia or

TABLE I  
*Ammonia Nitrogen and Pyruvic Acid Evolved from Digests  
of Di(glycylamino)propionic Acid with Dialyzed  
Aqueous Rat Tissue Extracts<sup>a</sup>*

Tissue	Ammonia N <sup>b</sup>	Pyruvic acid	Molar ratio of ammonia N to pyruvic acid
	<i>Micromoles</i>	<i>Micromoles</i>	
Kidney <sup>c, d</sup>	23	21	1.1
Intestinal mucosa	13	14	.9
Liver <sup>c</sup>	9	9	1.0
Spleen	5	6	.8

<sup>a</sup> Digests consisted of 1 cc. extract, equivalent to 333 mg. tissue and which had been dialyzed against distilled water for 6 hours at 5°C. prior to the digestion, plus 2 cc. 0.15 *M* borate buffer at pH 8.2, plus 1 cc. of either water or 25 micromoles of substrate. Incubation period was 4 hours at 37°C. Final pH. 7.9–8.0. Values given corrected for extract blanks. Boiled extracts are inactive.

<sup>b</sup> Neither di(chloroacetamino)propionic acid nor di(acetamino)propionic acid is split in these tissues under the experimental conditions.

<sup>c</sup> Neither glycine nor glycylglycine yields ammonia under these conditions. Fifty micromoles of DL-alanine yielded 6 micromoles of ammonia N, and 50 micromoles of glycyl-DL-alanine yielded 6 micromoles of ammonia N.

<sup>d</sup> Micromoles of ammonia N for pancreas 11, for brain 1, and for muscle 1.

pyruvic acid over the values for the control was noted in digests of di(chloroacetamino)propionic acid or of di(acetamino)propionic acid with extracts of rat kidney and liver. On the other hand, di(glycylamino)propionic acid was readily split by extracts of the rat tissues studied to yield both ammonia and pyruvic acid (Table I).

The molar ratio of ammonia N to pyruvic acid for each of the tissues studied is close to unity. In the case of the kidney digests, the micromoles of pyruvic acid formed from the substrate are close to the theoretical value of 25.

To observe the maximum quantities of ammonia and pyruvic acid produced in digests of di(glycylamino)propionic acid with aqueous rat kidney extracts the time course of the digestion was followed over several hours (Fig. 3). The maximum number of moles of either ammonia N or pyruvic acid is 1, and the molar ratio of ammonia N to pyruvic acid at each of the time intervals is close to unity. The finding

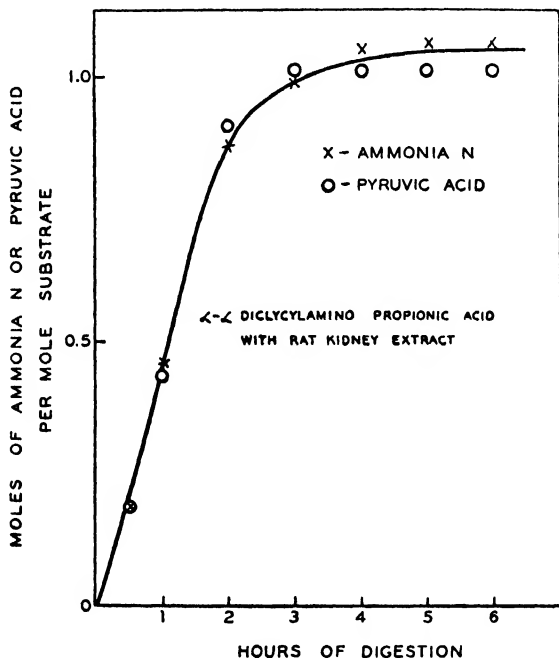


Fig. 3. Moles of ammonia N and of pyruvic acid evolved per mole of  $\alpha,\alpha$ -di-(glycylamino)propionic acid hydrochloride as a function of the time of digestion with aqueous dialyzed rat kidney extracts equivalent to 333 mg. tissue/cc. pH 8.1. Amount of substrate 25 micromoles.

that, on complete splitting of one mole of the peptide, one mole of pyruvic acid is formed, is confirmatory evidence of the structure of the peptide.

Fig. 4 illustrates the effect of pH on the splitting of di(glycylamino)-propionic acid in kidney extracts. The maximum splitting occurs at pH 8.0, and, at each pH of the digest studied, the molar ratio of ammonia to pyruvic acid is close to unity.

$\alpha,\alpha$ -Di(acetamino)propionic acid is stable in aqueous solution for at least several weeks and is resistant to enzymatic splitting under the conditions of our experiments. On the other hand,  $\alpha$ -acetamino- $\alpha$ -aminopropionic acid is rapidly and spontaneously split in neutral

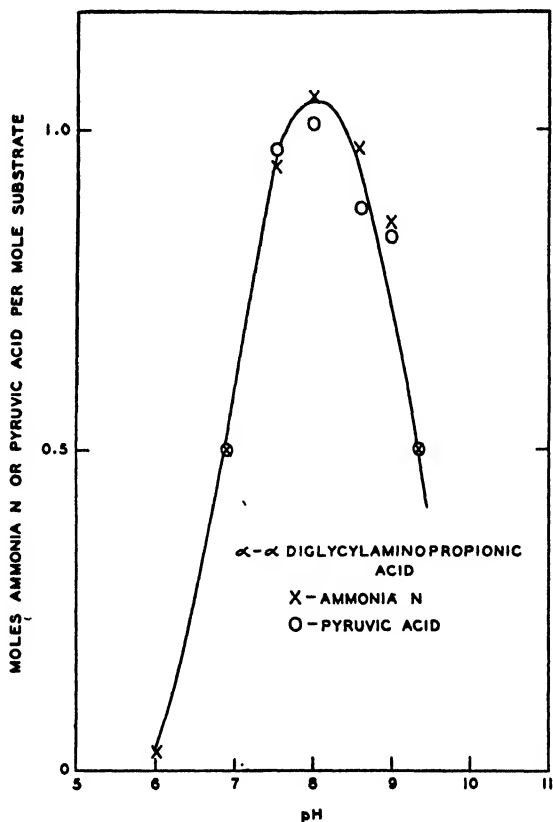


FIG. 4. Moles of ammonia N and pyruvic acid evolved per mole of  $\alpha,\alpha$ -di(glycylamino)propionic acid hydrochloride as a function of the pH of the digests with fresh rat kidney extracts equivalent to 333 mg. tissue/cc. substrate at 25 micromoles. Four-hour period of incubation at 37°C.

aqueous solution to yield equimolar quantities of pyruvic acid and ammonia (Fig. 5). The data in the figure refer to the moles of the products formed per mole of the substrate immediately after making up the fresh solution.

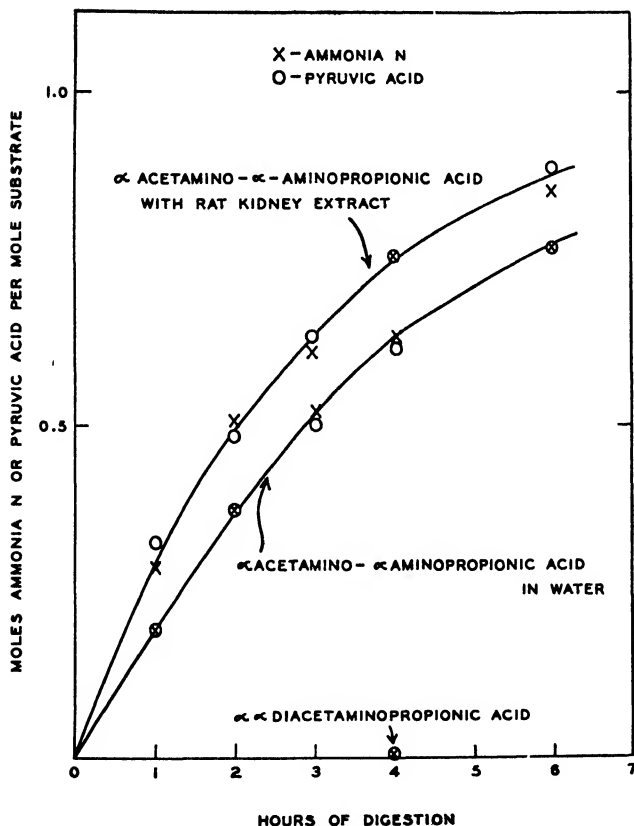
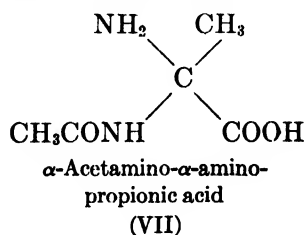
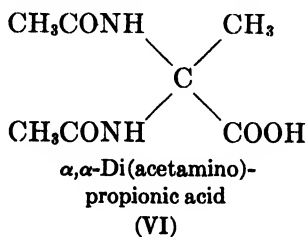


FIG. 5. Moles of ammonia N and pyruvic acid evolved per mole of  $\alpha$ -acetamino- $\alpha$ -aminopropionic acid spontaneously in aqueous solution and in the presence of aqueous dialyzed rat kidney extracts equivalent to 333 mg. tissue/cc. substrate at 25 micromoles, pH 8.0.

The relation between di(acetamino)propionic acid (VI) and acetaminoaminopropionic acid (VII) is represented as follows:



At each time interval the molar ratio of ammonia N to pyruvic acid is close to unity. In the presence of kidney extract, the amount of ammonia and pyruvic acid is significantly higher than that yielded by the spontaneous hydrolysis of the acetaminoaminopropionic acid. That an enzyme is involved in the digests of the kidney extract with the substrate is suggested by the fact that heated kidney extracts digested with acetaminoaminopropionic acid yielded no more ammonia

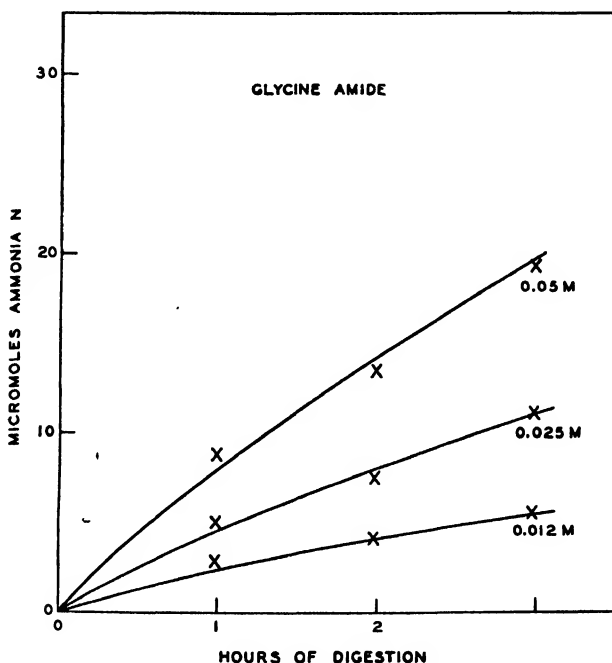


FIG. 6. Time course of the desamidation of glycine amide hydrochloride at various concentrations in digests with dialyzed extracts of rat kidney equivalent to 333 mg./cc. pH 8.1.

or pyruvic acid than that yielded by the spontaneous hydrolysis of the substrate in water.

Since it is possible that glycine amide may be one of the products formed by the enzymatic hydrolysis of di(glycylamino)propionic acid, the rate of desamidation of this compound was studied in digests with dialyzed extracts of rat kidney at pH 8.1 (Fig. 6). The rate of splitting of the amide is apparently independent of the concentration of sub-

strate, and in any event is relatively very slow as compared with the enzymatic splitting of di(glycylamino)propionic acid.

Centrifugation of aqueous rat kidney extract (333 mg. tissue/cc.) at 18,000 r.p.m. and at 5°C. for 2 hours yielded a pellet in which the greater part of the activity of the extract was concentrated. The

TABLE II

*Distribution of Activity in Splitting Di(glycylamino)propionic Acid and Glycine Amide in Fractions of Rat Kidney Extract<sup>a</sup>*

Fraction	Substrate			
	Di(glycylamino)propionic acid			Glycine amide
	Ammonia N	Pyruvic acid	Molar ratio of ammonia N to pyruvic acid	Ammonia N
Supernatant	<i>Micromoles</i> 8.3	<i>Micromoles</i> 8.2	1.0	<i>Micromoles</i> 1
Pellet suspended in distilled water <sup>b</sup>	22.4	21.6	1.0	10
Pellet resuspended in supernatant	21.0	19.8	1.1	6

<sup>a</sup> Experimental conditions identical with those in Table I.

<sup>b</sup> Suspension at same volume as original extract.

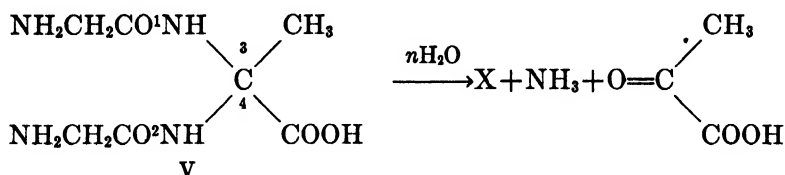
results are given in Table II. The molar ratio of ammonia N to pyruvic acid in all fractions remains close to unity.

## DISCUSSION

The molar ratio of ammonia to pyruvic acid formed in the course of the enzymatic splitting of di(glycylamino)propionic acid is important in the interpretation of the mode of such splitting. The maximum amount of pyruvic acid which can be formed from the complete splitting of one mole of di(glycylamino)propionic acid is obviously one mole. Depending upon the mode of splitting, however, the maximum ammonia N produced may be either one or two moles per mole of peptide, and the ratio of ammonia N to pyruvate formed during the course of the splitting may suggest not only the mode of splitting but also how many modes may be involved. The  $\alpha$ -amino groups on the



glycyl residues presumably are not hydrolyzable and do not furnish ammonia. Neither glycine nor glycyglycine furnishes ammonia after digestion with tissue extracts (Table I). The ammonia N is furnished by the nitrogen atoms attached to the tertiary carbon atom of the peptide, and, on complete hydrolysis in boiling hydrochloric acid, as noted above, two moles of ammonia N are formed per mole of peptide. Before pyruvic acid can be formed from the substrate, both linkages from the di(glycylamino) residues to the tertiary carbon must obviously be broken. The modes of splitting of di(glycylamino)propionic acid may be represented as follows:



(1) The substrate may be initially split at both peptide bonds, 1 and 2, yielding two moles of glycine, and one mole of  $\alpha,\alpha$ -di(amino)-propionic acid. The last-mentioned compound is unstable and would be expected to break down spontaneously to two moles of ammonia N and one mole of pyruvic acid. Molar ratio of ammonia N to pyruvate = 2.

(2) The substrate may be initially split at only one peptide bond, either 1 or 2, yielding one mole of glycine and one mole of  $\alpha$ -glycyl-amino- $\alpha$ -aminopropionic acid. The latter, in analogy with the  $\alpha$ -di-acetamino- $\alpha$ -aminopropionic acid described in Fig. 5, would be expected to be unstable and to break down spontaneously into one mole of glycine amide, one mole of ammonia, and one mole of pyruvic acid. Provided that the ammonia N contributed by the enzymatic desamidation of the glycine amide were negligible, the molar ratio of ammonia N to pyruvate = 1.

(3) The substrate may be initially split at both bonds 3 and 4 connecting the glycylamino residues to the tertiary carbon atom, yielding two moles of glycine amide and one mole of pyruvic acid. If the glycine amide is completely and rapidly split, the molar ratio of ammonia N to pyruvate = 2.

(4) The substrate may be initially split at only one bond between nitrogen and the tertiary carbon atom, that is, at either 3 or 4, yielding

glycine amide and glycyldihydroalanine. Provided that the ammonia N contributed by the enzymatic desamidation of the glycine amide is negligible, and since glycyldihydroalanine is rapidly hydrolyzed by dehydropeptidase I to yield equimolar amounts of ammonia and pyruvic acid (5), the molar ratio of ammonia N to pyruvate = 1.

The data given in Table I and in Figs. 3 and 4 show that, under the experimental conditions employed, di(glycylamino)propionic acid hydrochloride is rapidly hydrolyzed to yield a maximum of one mole of ammonia N and of one mole of pyruvic acid per mole substrate, and that, therefore, the molar ratio of ammonia N to pyruvate = 1. Modes of splitting 1 and 3 may be eliminated from consideration, leaving the choice between modes 2 and 4. The data in Fig. 6 show that the desamidation of glycine amide is weak and may for present purposes be neglected in the analysis of the data.

Mode 2 envisages the action of some enzyme on one of the two peptide bonds, leading to the formation of an unstable molecule, which spontaneously hydrolyzes to products which include equimolar amounts of ammonia and pyruvic acid (*cf.* Fig. 5). Superimposed upon this spontaneous hydrolysis would be some enzymatic activity similar to the situation described in Fig. 5. Kidney extracts are known to contain an active dipeptidase, but intestinal mucosa and pancreas contain a still more powerful dipeptidase activity, and it may be wondered why the activity of kidney extracts in splitting di(glycylamino)propionic acid is so much greater than that of either intestinal mucosa or pancreas (Table I). Another possible objection to mode 2 is the fact that peptides of glycine with amino acids containing a tertiary carbon atom, such as glycylaminoisobutyric acid, are relatively resistant to the action of dipeptidase (21). According to Bergmann *et al.* (21), substrates for dipeptidase must have a hydrogen atom on each  $\alpha$  carbon atom adjacent to the peptide bond, and neither di(glycylamino)propionic acid nor glycylaminoisobutyric acid nor, for that matter, glycyldihydroalanine satisfy this criterion. None of these three peptides possesses an asymmetric carbon atom. On the other hand, it should be pointed out that di(glycylamino)propionic acid is a type of substrate new in enzyme studies, and the possibility cannot be overlooked that crude tissue extracts may contain an enzyme system different from the classical dipeptidase system which may readily attack this substrate at one of the two peptide bonds.

Mode 4 envisages the action of an enzyme system, not known at the present time to occur in tissues, which presumably splits glycine amide from the substrate and which leaves glycyldehydroalanine to yield equimolar quantities of ammonia and pyruvate through the action of dehydropeptidase. An *in vitro* analogy to this possible initial reaction is that of Bergmann and Grafe (1), who heated a solution of  $\alpha,\alpha$ -di-(acetamino)propionic acid in glacial acetic acid and obtained  $\alpha$ -acetyldehydroalanine in satisfactory yield. This reaction has been confirmed (7). It would appear from this reaction that the di(acylamino) radicals in the di(acylamino)propionic acid peptides are not necessarily equivalent, and that the strength of the bonds holding them to the tertiary carbon atom may be greater in one than in the other.

From the present evidence, it is impossible to make a choice between modes 2 and 4. The possibility is not excluded that both may also occur simultaneously. Further investigations employing fractionation procedures on the tissue extracts used as the enzyme source for the present studies may explain this problem and provide the necessary answers. The present study may be considered essentially exploratory in nature. Whatever the nature of the enzyme systems may be which provide the initial attack upon di(glycylamino)propionic acid, it is evident that the substrate upon which they act must possess free  $\alpha,\alpha$ -amino groups on the molecule, since the corresponding  $\alpha,\alpha$ -di(chloroacetamino)propionic acid and  $\alpha,\alpha$ -di(acetamino)propionic acid are not split under conditions whereby the  $\alpha,\alpha$ -di(amino) compound is rapidly hydrolyzed (Table I).

The biologic significance of the present findings is of interest, for these findings suggest that the ketonic group of pyruvic acid may condense not only with one molecule of an amino acid amide (dehydropeptides) (22) but also with two molecules of an amino acid amide [di(acylamino)propionic acid peptides], and that both types of compounds are susceptible to enzymatic splitting, yielding, among other products, ammonia and the regenerated pyruvic acid in equivalent amounts.

#### SUMMARY

$\alpha,\alpha$ -Di(chloroacetamino)propionic acid was synthesized by an improved method, and from this compound the corresponding  $\alpha,\alpha$ -di(glycylamino)propionic acid hydrochloride was obtained by amination. Di(glycylamino)propionic acid possesses three ionizable groups:

$pK_1 = 1.8$ ;  $pK_2 = 8.1$ ; and  $pK_3 = 8.1$ . On hydrolysis with HCl, one mole of the peptide yields close to 2 moles of ammonia, 1 mole of pyruvic acid, and 2 moles of glycine.

Neither di(chloroacetamino)propionic acid nor di(acetamino)propionic acid is split in digests with rat tissue extracts, but di(glycylamino)propionic acid is split in digests with all the rat tissue extracts studied with the production of equimolar quantities of ammonia N and pyruvic acid. In descending order of relative activity, the tissues are: kidney, intestinal mucosa, pancreas, liver, spleen, brain, and muscle. Under the conditions studied, rat kidney extracts produce, as a maximum, one mole of ammonia N and one mole of pyruvate per mole of di(glycylamino)propionic acid. The optimal activity is at pH 8.0.

In contrast with the stability of the di(acylamino)propionic acids in aqueous solution,  $\alpha$ -acetamino- $\alpha$ -aminopropionic acid was found to be very unstable and rapidly hydrolyzed spontaneously into products which included a maximum of one mole of ammonia N and one mole of pyruvic acid per mole of peptide. The breakdown of this molecule was increased in the presence of aqueous kidney extracts.

Fractionation of rat kidney extract by means of high-speed centrifugation yielded a pellet in which the greater part of the activity in splitting di(glycylamino)propionic acid was concentrated. The molar ratio of ammonia N to pyruvic acid noted in digests of each of the fractions remained close to unity.

The possible modes of enzymatic splitting of di(glycylamino)propionic acid have been discussed.

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# Effects of X-Radiation on Sodium Thymus Nucleate <sup>1</sup>

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Received July 31, 1947

## INTRODUCTION

The study of the action of X-rays on isolated cellular components follows in logical sequence the observations of many workers on the profound effects of X-rays on the viability of cells, the morphology of chromosomes, the pattern of inherited characters, and the activity of certain viruses and enzymes. If X-rays can cause mutations, and if mutations involve chromosomal material, of which desoxyribonucleic acid is a prominent component, then it should be interesting to observe what effect X-rays have on the isolated nucleic acid itself. In undertaking such a problem it is necessary (1) to establish a criterion of "activity," loss of which is both amenable to measurement and directly related to some characteristic property of the nucleic acid molecule in what can be considered its native state, and (2) to have sufficient quantity of carefully defined, homogeneous material with which to work.

Unfortunately, the physiologic role of nucleic acid in cells is as yet obscure, and there exists no biologic or enzymatic test for assaying the degree by which one treatment or another may have altered the structure of nucleic acid so as to "denature" it. The best one can do is to isolate the material from cells in the mildest manner possible, designate as "native" the material thus isolated, and study the properties of both the native and the denatured material which has been altered by some physical or chemical means. When desoxyribonucleic acid is isolated from thymus gland by a modified Hammarsten-Bang procedure (1), as described herein, its most characteristic property in solution is its

<sup>1</sup> A preliminary notice of this work appeared in *Science* 105, 263 (1947).

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high anomalous viscosity. Since structural viscosity is a reflection of the asymmetry of a molecule, and in this case probably also an index of its polymerization, and can be easily and precisely measured, viscosity was the property of thymonucleic acid principally studied, before and after irradiation.

It has long been recognized that the viscosity characteristics of aqueous solutions of the sodium salt of thymus nucleic acid (TNA) vary considerably with the method of isolation employed. It was necessary for us to refine the method of preparation to obtain a large yield of highly polymerized, relatively salt-free TNA, from which we could take samples that would give reproducible results over a long period. Vilbrandt and Tennent (2) showed that the addition of acid or alkali degrades irreversibly the product obtained through Hammarsten's procedure, which involves no more drastic treatment than the use of salt and alcohol at pH 7 and at 0°C. By modifying the Hammarsten method suitably, we were able to obtain from thymus glands high yields of pure white TNA fibers, whose only appreciable contaminant was sodium chloride. This material is usually slightly hygroscopic; a typical dried sample gave the following analytical values: N, 14.0; P, 7.9; and ash, 12.01%. The N:P ratio, 1.82, corresponds closely to the theoretical value of 1.79 for a statistical tetranucleotide. Preliminary analyses of this preparation indicated the presence of inorganic chloride. Whether this chloride is an integral part of the nucleic acid fiber or a contaminant, remains for future investigation. The preparation was biuret-negative and in 2% sodium chloride was shown to be a homogeneous, monodisperse system by ultracentrifugation.

## EXPERIMENTAL PROCEDURE AND RESULTS

### *Preparation of Thymus Nucleic Acid (TNA)*

We have introduced a few modifications into the classic Hammarsten procedure; our procedure, due in considerable measure to the efforts of Dr. Charles E. Carter, in outline is as follows.

Fifteen pounds of calf thymus glands were cleaned, finely chopped, and homogenized in a Waring Blendor in cold, distilled water, and suspended in a final volume of about 15 l. of water. This material was left in the cold room overnight and then filtered through several layers of cheesecloth. The nucleoprotein was then precipitated from the filtrate as the calcium salt by the addition of about 20 cc. of 20% calcium chloride. The yield is greatly dependent on this step, an excess of calcium chloride causing the precipitate to redissolve. The precipitate settled slowly, and in 12 hours about  $\frac{3}{4}$  of the volume of the preparation could be siphoned off. The remaining supernatant was

centrifuged off in a Sharples supercentrifuge, the precipitate washed with cold water containing 1% calcium chloride, and redissolved in 1 l. of 10% sodium chloride. Once in solution, solid sodium chloride was added to saturation, and the resulting solution was poured into 12 l. of cold, saturated sodium chloride. This solution was allowed to stand in the cold for 3 days to dissociate the nucleic acid from its protein component. It was then filtered, with suction, with the aid of Hyflo supercel, from which a clear, highly viscous filtrate was obtained. TNA was precipitated from the filtrate with cold 95% alcohol.

We found the yield to be greatly increased if we squirted the TNA-containing filtrate into large beakers of alcohol through 100-cc. syringes. As soon as the stream of filtrate under pressure hit the alcohol, TNA precipitated out in long fibers, occluding some air bubbles in the process, so that after a few minutes standing the TNA precipitate contracted and rose to the surface of the alcohol, while the salt streamed to the bottom of the container. The fibrous precipitates were collected, pooled, and washed several times in 70% alcohol, then run up through 80, 90, 95%, and absolute alcohol, and finally washed with ether. The product was white and fluffy and dried very quickly in air in the form of long white fibers. It was stored in a dry bottle but not in a desiccator, since a certain amount of water appears to be necessary to maintain the integrity of the fiber mass. When the air-dried preparation was stored in a vacuum desiccator over phosphorus pentoxide, a considerable change in physical properties resulted. Aqueous solutions made up from such desiccator-dried preparations had a pH of about 4, as contrasted with the normal pH, 6.5, and a decidedly lower viscosity. Consequently, this dried sample was not used for any viscosity measurements; it was used only to determine the extinction coefficient of the preparation, which constant was then utilized to calculate the concentration of subsequent solutions of TNA made up from material containing variable amounts of water. The curve of extinction coefficient *vs.* wavelength for a solution containing 0.033 mg./100 cc. was measured with a Beckman ultraviolet spectrophotometer at maximum absorption,  $\lambda = 2600\text{\AA}$ . The value for the extinction coefficient was 20.6 over a concentration range of 0.01–0.05 mg./100 cc. Unknown concentrations were subsequently evaluated by diluting to the proper range and dividing optical density at 2600 $\text{\AA}$  by 20.6. Cuvettes with 1 cm. path were employed.

For making up solutions, the general procedure was to dissolve a weighed amount of TNA in water or salt solution, draw out the air bubbles with moderate evacuation, and dialyze the solution against 6 l. of either distilled water or the required salt solution from 12 to 36 hours. This was necessary to insure the presence of a uniform amount of salt. The relative viscosity of solutions made up in this way was uniformly high and anomalous, *i.e.*, greatly dependent on the velocity gradient or the applied pressure. Standing for 24 hours had no effect on the viscosity function, and dialyzing only increased the absolute magnitude of the viscosity by reducing the salt content. The extent of the anomaly increases markedly with small increments of concentration, (*cf.* 6) and it was found inconvenient under present conditions to work with any concentrations above 0.3%. The viscosity of the TNA solutions was found to be insensitive to changes in pH within the range of 6.8–9.5. A slight drop in viscosity was noted upon acidification below pH 6.5.

Bingham-Jackson type viscometers (3) were used, and the viscosity was measured under different applied pressures in a water bath at 30°C. The pressures used varied



from 2 to 24 cm.  $H_2O$  and were constant during the measurement to within 2 mm.  $H_2O$ . Viscosity-pressure curves were extremely anomalous, and both the height and the shape of the viscosity-pressure curves changed with changing concentrations of TNA. Consequently, for purposes of better comparison, following Edsall and Mehl (4), viscosity was plotted against mean velocity gradient, or  $\beta$ , according to Kroepelin (5), where  $\beta = 8V/3\pi r^2t$ , since this function is independent of the measured pressure and dependent only on the time of flow and the dimensions of the capillary involved (6).

### *Immediate Effect of X-Radiation*

X-radiation was delivered at 5,600 r/minute by a standard dual X-ray machine operating at a peak voltage of 180,000 volts, with 20 ma current through each tube. The sample was placed midway between the two tubes, whose targets were 23 cm. apart.

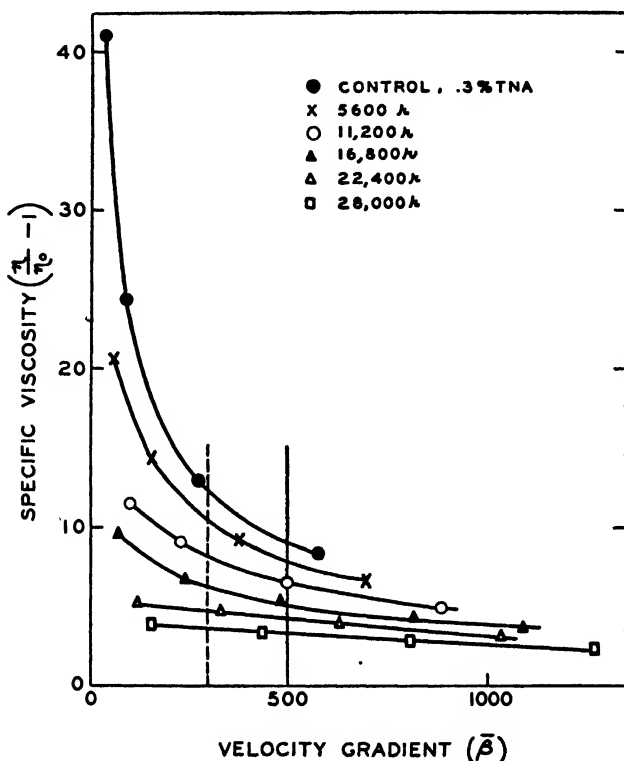


FIG. 1. Effect of various doses of X-rays on the structural viscosity of TNA. Concentration, 0.3% in 2% NaCl solution.

The anomalous viscosity noted in 0.3% solutions of TNA in 2% NaCl is almost completely lost by doses of X-radiation above 22,400 r (4 minutes). The relative viscosity of the X-rayed sample is not much higher than water and completely independent of the applied pressure or of the velocity gradient. This infers a large decrease in molecular asymmetry over the unirradiated control. The minimum amount of X-radiation needed to reduce almost completely the structural viscos-

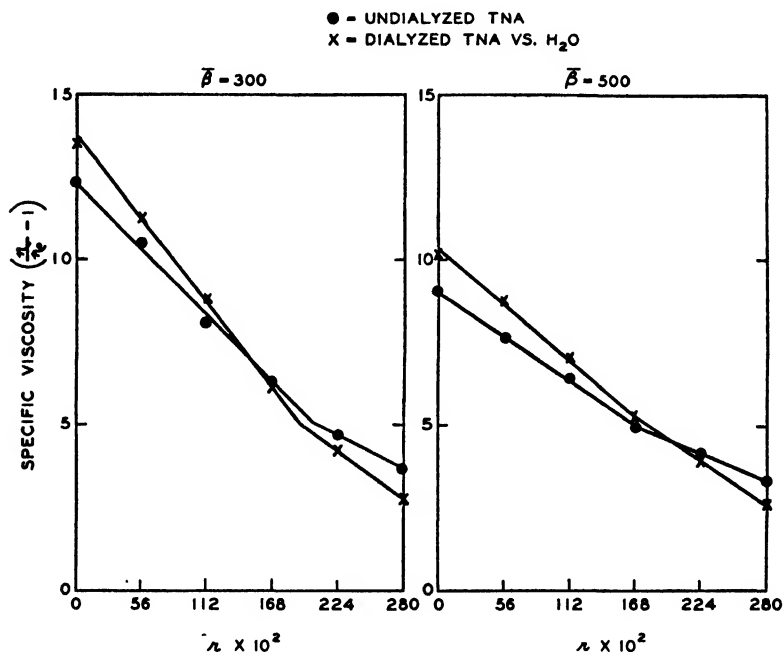


Fig. 2. Dosage curves of TNA taken at given values of  $\beta$  from Fig. 1.

ity was 22,400 r, as demonstrated by the curves in Fig. 1. Greater amounts of irradiation merely lowered the position of the lowest straight lines. To obtain a dosage curve from these data, relative viscosities at constant  $\beta$  were plotted against dose of radiation for two widely separated values of  $\beta$  taken from the points intersected by the two vertical lines in Fig. 1 (Fig. 2). Up to 4 minutes of irradiation the drop is linear, but after the anomaly has disappeared, the drop in viscosity with dosage becomes exponential, similar to the relationship found by Sparrow (7).

*Spontaneous Decrease in Viscosity*

Surprisingly, the drop in viscosity of TNA solutions initiated with X-rays continued to be expressed after cessation of the irradiation for a period of about 8 hours, when it leveled off and approached the spontaneous drop of the control (8). When a reading of relative viscosity was taken as soon as possible after irradiation (10–30 minutes being allowed for the attainment of temperature equilibrium) and another reading taken 4 hours later, a drop of from 20 to 40% of the first reading was noted. The unirradiated control was relatively stable, its

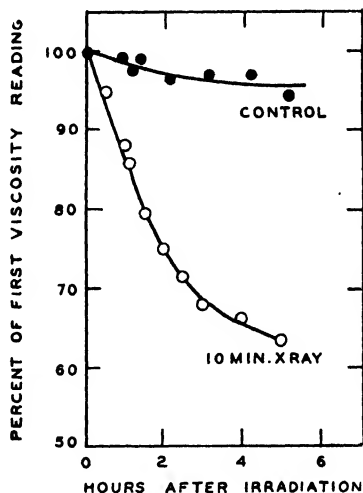


FIG. 3. Rate of decrease in viscosity of TNA in aqueous solution after the end of the irradiation period; 0.2% TNA, dialyzed against water; dosage, 56,000 r.

viscosity decreasing from 0 to 5% in 4 hours. For this reason, the measurements of viscosity *vs.* pressure, of the irradiated TNA solutions, and all dosage curve measurements were performed 12 hours after the end of irradiation, after the solutions had reached a steady value. This subsequent fall with time after irradiation is shown in Fig. 3, in which the percentage of the first reading taken after the irradiation (usually 30 minutes after) is plotted against increasing time of standing. The presence of salt had no effect on this subsequent drop; whether the solutions were dialyzed against distilled water or salt made no difference as far as the per cent drop with time was concerned, although the absolute value of the control was, of course, higher in water solution

(*cf.* 6). Added protein (1% egg albumin final concentration) protected against the effect of X-rays, since the initial fall in viscosity was about 20% that in the absence of the protein (*cf.* 9), but it did not affect the rate of subsequent drop with time of standing. The viscosity of TNA solutions prepared by dissolving the nucleate in previously irradiated water did not diminish appreciably with time of standing. From this one can tentatively conclude that, although the action of X-rays is probably both directly on the molecule itself and indirectly through "activated" water molecules, the subsequent drop with time of standing is a reflection only of internal change in the molecule itself, concomitant with the establishment of a new equilibrium.

An interesting difference emerges in the effect of ultraviolet radiation and of X-radiation on sodium thymus nucleate, for, whereas both forms of radiation produce a decrease in the viscosity of solutions of the nucleate, the drop induced by the former is constant following cessation of irradiation (10) while the drop induced by the latter continues after irradiation has ceased.

### *Effect of Temperature*

Interesting in this respect is the effect of temperature on the phenomenon. A 0.2% solution of TNA dialyzed against water was divided into 3 aliquots: one was not irradiated; one was irradiated for 3 minutes at room temperature; and one was irradiated for 3 minutes while packed in ice with an inside measured temperature of 5°C. The

TABLE I  
*Effect of Temperature on Viscosity Changes Induced in Sodium Thymus Nucleate by X-Radiation*

Treatment	Viscosity at 30°C.		
	10 minutes after irradiation <sup>a</sup> (1)	After standing 11 hours at 25°C. (2)	After standing 12 hours at 5°C. (3)
	Relative viscosity	Relative viscosity	Relative viscosity
Control	13.3	13.4	13.0
X-radiated at 25°C.	7.9	4.8	6.7
X-radiated at 5°C.	8.8	5.1	7.1

<sup>a</sup> 16,800 r in 3 minutes.

time from the end of irradiation to the beginning of the viscosity measurement at 30°C. was measured with a stop watch, and a reading was taken of the relative viscosity of each sample exactly 10 minutes after the end of the irradiation. The sample irradiated at room temperature had a viscosity, relative to the control, only 7% lower than the one irradiated while cold. Immediately after the irradiation, stoppered aliquots of each of these samples were placed in the icebox at 5°C., and at room temperature, for determinations of the effect of temperature on the subsequent decrease with time. Table I gives the data. The viscosity of both samples decreased approximately the same amount, when standing at room temperature and also when standing at 5°C. But in each case the subsequent drop with time was suppressed by about half in the samples that stood in the cold. This would indicate that the fall in viscosity with time after irradiation was more sensitive to temperature than the direct action of X-rays on the solution of TNA. Fig. 4 shows the course of the viscosity fall for a 0.2% TNA solution irradiated at 25° and 5°C.

### *Sedimentation Data*

The drop in structural viscosity, upon irradiation with X-rays reflects a marked change in the asymmetry of the particle. But still two possibilities are open, either the original nucleic acid fiber has been broken into smaller fragments or the original long nucleic acid fibers have associated in rounder, more asymmetrical units. To answer this problem, it was necessary to obtain data from an independent type of physical measurement.<sup>3</sup> Three concentrations were run, each, of the unirradiated sample and of an aliquot which had been X-irradiated for 30 minutes (168,000 r), both in the presence of 2% salt. Pictures were taken at 30-minute intervals with the Phillipot schlieren lens system over a period of 2.5 hours. The sedimentation was run at 25°C. In Fig. 5 are shown the tracings for each concentration taken after 1.5 hours. There is a marked difference in the behavior of the two systems; the unirradiated control moves to the bottom of the cell as an essentially monodisperse system, while the irradiated sample shows a distribution indicating the presence of many particle sizes. The sedimentation con-

<sup>3</sup> We are greatly indebted to Dr. Gerson Kegeles and Mr. E. Hanson, of the Department of Chemistry of the University of Wisconsin, for making 6 sedimentation velocity runs for us in their ultracentrifuge.

stants calculated by Dr. Kegeles were; for the control, 11 S; for the irradiated, roughly 5 S. The sedimentation for the control was a linear function of the concentration, and 11 S represents an extrapolation to 0 concentration; but for the X-rayed sample no such relationship could be obtained, and 5 S represents the average constant. These data

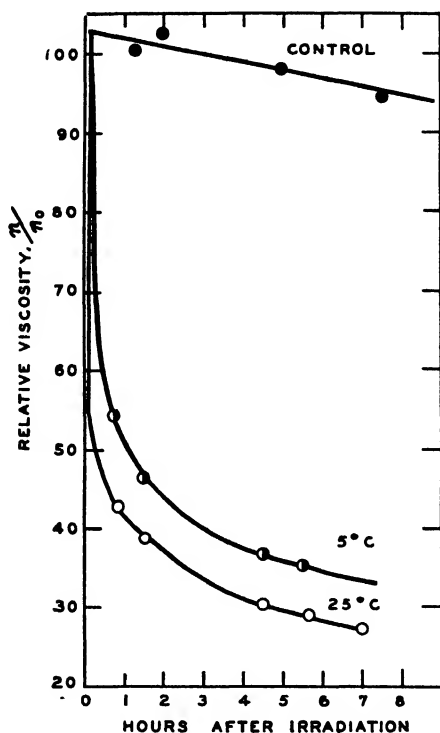


FIG. 4. Effect of temperature on the decrease of viscosity of TNA after irradiation. Dosage, 56,000 r; nucleate concentration, 0.2%; viscosity measured at 30°C.

suggest that X-radiation causes a breaking of the nucleic acid particles into smaller fragments.

#### *Chemical and Enzymatic Studies*

In order to test the possibility that inorganic phosphate or ammonia or nucleotide residues might have been split off the TNA molecule upon irradiation, 5 cc. aliquots of a 0.3% solution of TNA before and

after irradiation were pipetted into bags of dialyzing tubing which were immersed in 20 ml. of distilled water in 50 cc. centrifuge tubes and placed in an incubator at 37°C. (11). After a period of 4 hours, the

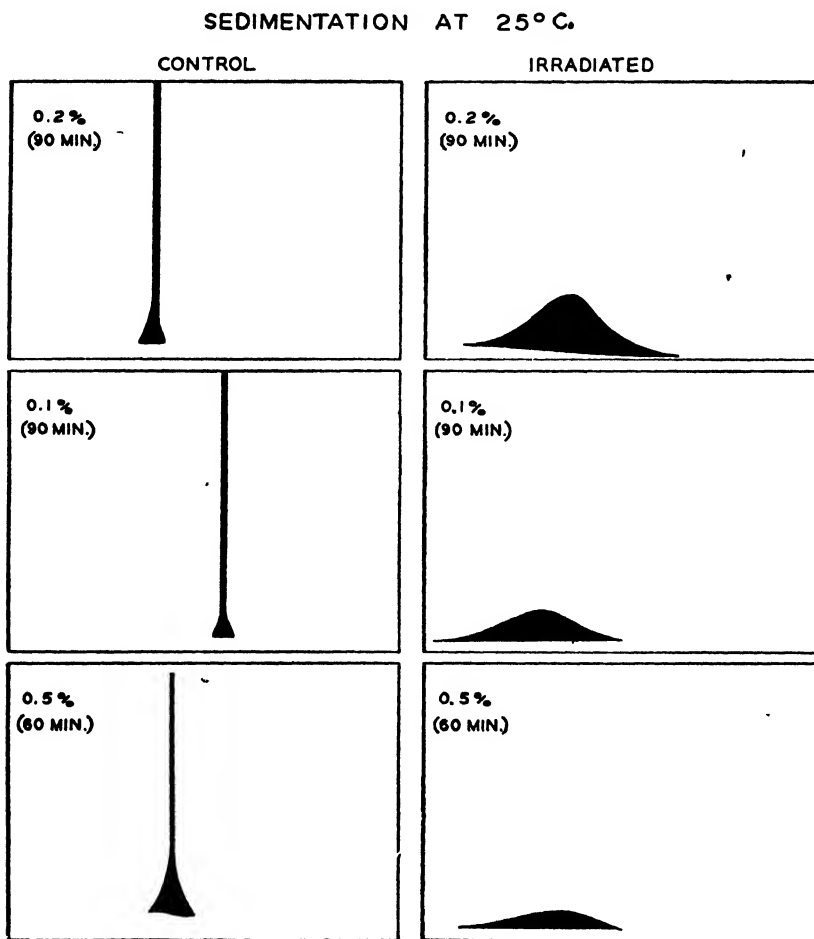


FIG. 5. Sedimentation diagrams of irradiated and control TNA solutions in 2% sodium chloride at 25°C. Tracings indicate sedimentation after 90 and 60 minutes.

outside dialyzate was tested chemically for ammonia and phosphate, and spectrophotometrically for components possessing an absorption at 2600A. In the case of both control and irradiated material, tests for

ammonia and both organic and inorganic phosphate were completely negative, and the amount of absorbing material which passed through the bag did not amount to more than 1% of the total material. From these experiments, two conclusions were drawn: (1) That the fragmentation of TNA by X-rays does not involve the splitting of the nucleate to release ammonia, phosphate, or purine residues; and (2) that the molecular size, on a spherical basis, of the resulting fragments is over 10,000 (assuming about 20 Å to be the average diameter pore of the cellophane membrane). The data are summarized in Table II.

TABLE II  
*Comparison of Properties of TNA before and after Irradiation*

Properties	Before	After
Viscosity	Anomalous, relatively stable	Newtonian, falls with time
Flow birefringence <sup>a</sup>	+++	0
Sedimentation constant	11	ca. 5
Extinction coefficient	20.6	20.6
Refractive index (0.11%)	1.3363	1.3362
Dialyzable phosphate	0	0
Dialyzable ammonia	0	0
Enzymatic susceptibility <sup>b</sup>	High	Equally high
pH	6.5	6.0
Alcohol precipitability	+	—
Acid precipitability	+	+

<sup>a</sup> As estimated between crossed Polaroid prisms (*cf.* 4).

<sup>b</sup> Measured by rate at which dialyzable products (through cellophane) are formed by the action of purified (McCarty) desoxyribonuclease + Mg<sup>++</sup>.

The absorption spectrum of the irradiated material, as measured in the Beckman spectrophotometer, corresponds closely to that of the unirradiated control. Streaming birefringence of flow is, as Sparrow (7) also found, almost completely abolished on irradiation. No significant change in either titratable acid groups or acid precipitability could be detected. However, the ability to precipitate out in 95% alcohol in the presence of salt is completely lost in the irradiated material. Slight changes in pH were occasionally noted, the X-rayed samples sometimes dropping as much as 0.5 pH unit below the control. But there was no regularity about this observation; and since nucleic acid exhibits practically negligible buffering power in the range of pH 6–7, these changes were considered insignificant.



Tests were made to compare the enzymatic susceptibility of the TNA solutions before and after irradiation. Samples of control and irradiated TNA were incubated with McCarty's desoxyribonuclease plus  $Mg^{++}$  according to the technique described by Carter and Greenstein (11). There was no detectable difference in the rate of hydrolysis of the two materials. In the same way, extracts of rat spleen, high in desoxyribonuclease activity, were used to detect a difference in the substrates. Here again, both substrates were hydrolyzed to the same extent, and their hydrolysis was accelerated to the same extent by the addition of NaCl. These studies indicate that the chemical and enzymatic tests which are applicable to solutions of sodium thymus nucleate are insensitive to the physical state of aggregation of the particle, at least within the range of particle sizes encountered in 20-30 minutes of X-radiation.

As far as the mechanism of the action of radiation on nucleic acids is concerned, that is, whether direct or indirect, *etc.*, we can say very little. Certainly there is a protective action exhibited by the addition of egg albumin to the solution of thymus nucleic acid. This, according to Lea, would indicate an indirect effect, mediated by some type of "activated" water molecules. That the effect is due to the presence of hydrogen peroxide in the irradiated solvent is made improbable by the fact that the addition of peroxide, even in concentrations ( $10^{-2}$ - $10^{-5}$  *M*) that far exceed those postulated for activated water has no effect on the viscosity of aqueous solutions of thymus nucleic acid.

#### ACKNOWLEDGMENT

We are indebted to Dr. J. M. Gonçalves for his stimulating advice and suggestions, and to Mr. Henry Meyer for assistance in the X-ray measurements.

#### SUMMARY

The effect of X-rays on aqueous solutions of sodium thymus nucleate is apparently to break up the long, fibrous particle into shorter fragments of variable dimensions. The passage from a state of monodispersion to a state of wide polydispersion continues, after the actual irradiation has stopped, for several hours until a new equilibrium is reached. The extent of the change, or the level of that new equilibrium depends on the dose of roentgens delivered and is independent of the time over which that dose is given. The chemical properties and

susceptibility to desoxyribonuclease- $Mg^{++}$  of sodium thymus nucleate are essentially independent of the state of polymerization or dispersion as altered by X-rays.

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# Chemical and Electrophoretic Studies of Fibrinogen Prepared by Various Methods

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Received June 9, 1947

## INTRODUCTION

Many of the procedures used in the study of blood coagulation require, at some point, the addition of fibrinogen which is free of other active coagulation factors. This fibrinogen must, therefore, be free of prothrombin and thromboplastin, and as nearly as possible a pure homogeneous protein solution of constant concentration. It should be sufficiently stable to avoid the necessity for a fresh preparation for each experiment.

Recent methods of preparing fibrinogen solutions have involved two steps: (a) removal of prothrombin from plasma and (b) precipitation of the fibrinogen from the prothrombin-free plasma by salts or alcohol.

Removal of prothrombin has been accomplished by means of various adsorbents. Bordet and Delange (1) used tricalcium phosphate, Smith *et al.* (2) used magnesium hydroxide, while Ferguson (3) and Quick (4) used aluminum hydroxide.

Florkin (5), and Chargaff and Bendich (6), used saturated sodium chloride as a precipitating agent for the fibrinogen. Florkin (5) specified pH limitations and temperature control (2°C.) for handling the precipitate to reduce denaturation of the protein. Ammonium sulfate was used by Ferguson (3) and Quick (4) to precipitate the fibrinogen. Jaques (7) used Florkin's pH control and temperature range but employed 2 *M* potassium phosphate buffer as a precipitating agent.

The alcohol precipitation method of Cohn (8), which has been employed in a modified form by Seegers *et al.* (9), produces a fibrinogen which has already been studied chemically and electrophoretically by these workers. The method is one which requires the use of a cold room and is not feasible as an ordinary laboratory procedure.

The salt precipitation procedures are those commonly used in laboratory preparation of fibrinogen for use in studies of blood coagu-

lation. Since the use of fibrinogen is ultimately involved in all such studies, it appeared to us of interest to study these already established procedures, with the object of determining which yielded the most desirable product. No attempt has been made to develop a new procedure for preparing fibrinogen, and the precipitation procedures previously described were followed as closely as possible.

Three methods of salt precipitation were studied: potassium phosphate as described by Jaques (7); ammonium sulfate as described by Ferguson (3); and sodium chloride as described by Chargaff and Bendich (6). The chemical and electrophoretic properties of each of these preparations were used as criteria of their relative purity. All preparations were made from a single lot of human plasma.

## METHODS AND MATERIALS

### *Plasma*

The plasma used in these preparations was obtained from 1,500 ml. of human blood discarded by the blood bank because of positive Kahn tests. It had been drawn by venepuncture and mixed in a sterile bottle with 1/9 volume of sodium citrate. The plasma was obtained by centrifuging. It was then treated according to the method of Quick (4) with 0.1 its volume of aluminum hydroxide<sup>1</sup> to remove prothrombin. The aluminum hydroxide was removed by centrifuging and the adsorption repeated. The plasma then gave no clot on addition of thromboplastin and calcium chloride.

This plasma was divided into lots of 200 ml. each and stored at  $-20^{\circ}\text{C}$ . until used.

### *Citrated Saline*

This solution was prepared to contain 0.15 *M* sodium chloride and 0.00765 *M* sodium citrate.

### *Preparation of Fibrinogen Solutions*

All operations were carried out at  $2^{\circ}\text{C}$ . and centrifuging was done in cups packed with crushed ice.

*a. Phosphate Precipitation.* This was carried out according to the procedure described by Jaques (7). Following each precipitation the total volume of solvent in which the fibrinogen was taken up was reduced so that the final preparation was contained in a volume of 50 ml. The final fibrinogen precipitate was dissolved in 0.15 *N* sodium chloride containing 0.00765 *M* sodium citrate, instead of the 0.25 *M* phos-

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<sup>1</sup> Wyeth's Amphogel, without flavor. This product conforms to the U.S.P. standards for aluminum hydroxide gel. We are indebted to Dr. Alfred Barol, Director of the Wyeth Institute of Applied Biochemistry, for supplies of this material, and for information regarding its properties.

phate buffer used for dissolving the precipitate following the first and second precipitations.

*b. Ammonium Sulfate Precipitation.* This was done using the reagents described by Ferguson (3), but following the method of precipitation as described by Jaques (7) for the phosphate preparation. The fibrinogen was precipitated three times, washed twice with 0.5 saturated ammonium sulfate and dissolved in a final volume of 50 ml. of citrated saline.

*c. Sodium Chloride Precipitation.* This preparation was made using the reagents described by Chargaff and Bendich (6) but, again, following the method of precipitation employed for the phosphate precipitation. As in the previous precipitations the final precipitate was dissolved in 50 ml. citrated saline.

Each preparation was divided into three lots of 10 ml., 20 ml., and 20 ml. The 10 ml. lot was dialyzed against 2 liters of citrated saline overnight and used for chemical studies, and the two lots of 20 ml. each were reserved for electrophoretic studies.

### *Chemical Studies*

*Total Protein.* Total protein was calculated from the total nitrogen determined in 0.5 ml. of the fibrinogen solution by the micro Kjeldahl procedure followed by Nesslerization.

*Fibrinogen.* Fibrinogen was determined by converting it to fibrin and determining the protein nitrogen content of the clot. One-half ml. of the fibrinogen which had been dialyzed against citrated saline was mixed with 0.1 ml. of thrombin solution,<sup>2</sup> containing 25–30 units of thrombin/ml. After standing for 1 hour at room temperature, the clot was removed, washed with 0.15 *N* sodium chloride, dried with filter paper and analyzed for nitrogen by the same procedure as for total protein.

The recent work of Ferry and Armstrong (10) indicates that the yield of fibrin obtained from a solution of fibrinogen is dependent on the conditions under which the clotting takes place. Our studies were completed before their data were available. Since, however, the same conditions of analysis prevailed for each preparation, we consider that our data are indicative of the relative concentrations of each preparation.

*Prothrombin Activity.* Tests for prothrombin activity were made by Quick's method (11).

### *Electrophoresis Studies*

All preparations were studied in both a sodium phosphate buffer, ionic strength 0.1, containing 0.00765 *M* sodium citrate, at a pH of 7.4; and a buffer consisting of 0.1 *N* sodium diethylbarbiturate and 0.02 *N* diethylbarbituric acid at pH 8.6 (12), also modified to contain 0.00765 *M* sodium citrate (13). The fibrinogen preparations were dialyzed in 18/32 in. cellophane tubing for 18 hours against 1,000 ml. of buffer, followed by 24 hours dialysis against 2,000 ml. of the buffer. The buffer used for the final dialysis was used to fill the electrode vessels of the apparatus. As a routine procedure all samples were packed in ice and centrifuged for 20 minutes at 2,200 r.p.m.

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<sup>2</sup> Parke, Davis and Co., Thrombin Topical; 1 ampoule being made up to a volume of 200 ml. with 0.15 *N* sodium chloride. We are indebted to Mr. Eugene C. Loomis for generous supplies of this material.

Electrophoretic analyses were carried out in the apparatus described by Tiselius (14), equipped with the Toepler schlieren optical arrangement as modified by Longworth (15). A single section cell of 11.0 ml. capacity was used. Electrophoresis was allowed to proceed at approximately 5.3 volts/cm. for 180–240 min. depending on the nitrogen concentration of the material. The fraction of the total protein contributed by each of the components was calculated from the descending pattern as the ratio of each component area to the total area (exclusive of the  $\epsilon$ -boundary). The method suggested by Tiselius and Kabat (16) was used for allocating the area of each peak.

## RESULTS

The data obtained from this study are presented in Table I. From chemical studies of the preparations it was found that, while ammonium

TABLE I  
*Analytical Data on Fibrinogen Preparations*

Method of precipitation	Chemical				Electrophoretic					
	Protein			Prothrombin time	Buffer	No. of components	Mobilities (cm. <sup>2</sup> /volt/sec. $\times 10^6$ )		Per cent total area	
	Total	Fibrin	Fibrin Total $\times 100$				Major	Minor	Major	Minor
	g./100 ml.	g./100 ml.		min.						
Potassium phosphate	0.504	0.510	100	45	Veronal phosphate	1 1	2.30 2.17	—	100 100	—
Ammonium sulfate	0.546	0.514	94	>60	Veronal phosphate	2 2	2.18 2.52	2.93 3.38	94 96	6 4
Sodium chloride	0.214	0.193	90	>60	Veronal phosphate	2 1	2.37 1.98	3.43 —	84 100	16 —

sulfate precipitation yielded the greatest total concentration of protein, only 94% of this protein clotted on the addition of thrombin. Sodium chloride precipitation gave a poor protein yield, of which only 90% clotted with thrombin. Phosphate precipitation gave a quite satisfactory yield of protein which was 100% clotted with thrombin. It is not possible to give the yield of fibrinogen as percentage of the amount present in the original plasma, since the sample of the original plasma reserved for determination of fibrinogen was accidentally lost. In no case was prothrombin present in any significant amount.

The electrophoretic studies corroborate the chemical findings. While the time of electrophoresis may not have been long enough to show complete resolution of fibrinogen from all impurities (17), the electrophoretic studies do indicate a considerably greater degree of purity in

the phosphate preparation as compared to the ammonium sulfate and sodium chloride preparations.

In the veronal buffer the phosphate fibrinogen (Fig. 1a) showed a single component, indicating complete homogeneity. The ammonium sulfate and sodium chloride preparations (Figs. 1b and 1c) both contained a major component with approximately the same mobility as that obtained for the phosphate preparation, with a minor component having a higher mobility. The mobilities of these major components and the single component of the phosphate preparation agree satisfactorily with Dole's (18) mobility for fibrinogen ( $2.14 \pm 0.25$ ) in unfractionated human plasma in this buffer.

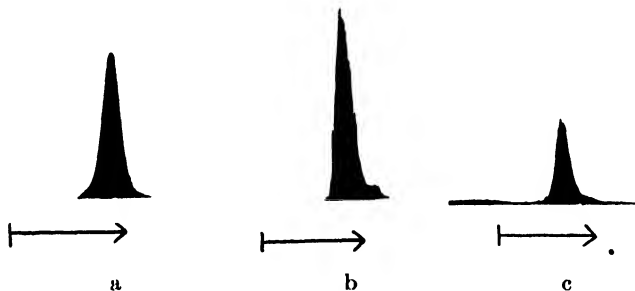


FIG. 1. The electrophoretic pattern of fibrinogen preparations in veronal buffer; descending pattern; (a) precipitated with potassium phosphate, (b) precipitated with ammonium sulfate, (c) precipitated with sodium chloride.

In the phosphate buffer both the phosphate and sodium chloride preparations showed a single component while the ammonium sulfate preparation again showed two components. During the course of dialysis of the sodium chloride preparation against this buffer a heavy precipitate developed. A very small area appeared in the electrophoresis pattern, as compared to that in the veronal buffer, indicating a smaller amount of protein present. It seemed probable, therefore, that the impurity present at the higher pH of the veronal buffer was lost by precipitation in the phosphate buffer at pH 7.4.

#### SUMMARY

A comparative study has been made of the properties of fibrinogen prepared from human plasma by the use of previously described methods of salt precipitation. These methods involved the use of



potassium phosphate, ammonium sulfate, and sodium chloride, respectively, as precipitating agents.

Of these three procedures, precipitation with potassium phosphate yields a product which is superior to that obtained by precipitation with either ammonium sulfate or sodium chloride.

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# The Oxidase of the Olive

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Received June 18, 1947

## INTRODUCTION

In the pickling of ripe olives by the California procedure, a dark brown to black color is formed by oxidation, and in the pickling of green olives by the Spanish process occasionally an undesirable brown or gray color induced by oxidation occurs. Both oxidations are due in part to enzyme activity. Therefore, it is of some interest to know something of the nature of the oxidase of this fruit.

While much has been published on the oxidizing enzymes of various plants, fruits and vegetables, we were unable to find any report in the literature on olive oxidase other than a brief note by Cruess and Fong (2) to the effect that olive tissue gave positive reactions with benzidine indicator alone for "complete oxidase"; with dilute  $\text{KI-CH}_3\text{CO}_2\text{H-starch}$  solution for "organic peroxide," and with benzidine plus  $\text{H}_2\text{O}_2$  for peroxidase. Sumner and Somers (6) in their recently revised book (1947) give an excellent review of oxidizing enzymes, and present comprehensive bibliographies on the several classes of these enzymes.

Samisch (5) states that phenolases are oxidases which have the power to catalyze the oxidation, by molecular oxygen, of phenols or their simple derivatives containing free OH groups. In general, the phenolic substances naturally occurring in fruit tissues are very likely to be those containing an orthodihydroxy phenolic grouping. This is particularly true of the olive, as a very strong qualitative test for this grouping is obtained with dilute  $\text{FeCl}_3$  plus  $\text{NaHCO}_3$ . Also, Cruess, 1931, showed that, on alkaline hydrolysis, the bitter principle of the olive yields a large amount of crystalline caffeic acid, an orthodihydroxy compound.

## EXPERIMENTAL

### *Qualitative Tests*

Qualitative tests with oxidase indicators were made upon the cut surface of fresh olives, on juice diluted with 2-3 volumes of  $\text{H}_2\text{O}$ , and

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on the purified enzyme. For this purpose the purified enzyme was prepared by pitting the fresh olives, grinding in a mortar, adding 3 volumes of acetone and some clean sand, grinding, filtering, and repeating this cycle 4 more times, air drying the solid residue, dissolving the enzyme in 1.5%  $\text{NaHCO}_3$ , filtering, and neutralizing to about pH 5 with 5%  $\text{HAc}$ .

The cut surface and the diluted puree gave strong positive tests for "complete oxidase" (Onslow's "oxygenase") or phenolase with dilute guaiacol solution; also for "peroxidase" with dilute  $\text{H}_2\text{O}_2$  and benzidine or  $\text{H}_2\text{O}_2$  and dilute guaiacol solution. But, unexpectedly, the purified enzyme solution gave negative tests, not only for complete oxidase but also for peroxidase; *i.e.*, with benzidine and guaiacol alone, and these reagents with  $\text{H}_2\text{O}_2$ . With a trace of added catechol, or with imperfectly purified oxidase, positive phenolase ("oxygenase" and "complete oxidase") were obtained with the purified enzyme, using benzidine and guaiacol without and with added  $\text{H}_2\text{O}_2$ . In order to prepare an oxidase free of catechol-containing substances, the olives were ground with sand in a mortar with acetone. Unfortunately this was not realized until our quantitative tests had been completed, indicating that the purified oxidase preparation used in our quantitative tests contained sufficient catechol-containing substance to give a positive reaction with guaiacol and  $\text{H}_2\text{O}_2$ .

Therefore, the evidence is quite clear that the olive contains a phenolase rather than a peroxidase, because, in the absence of orthodihydroxy phenolic substances, the purified enzyme fails to give positive tests for peroxidase (coloration of oxidase indicators in the absence of orthodihydroxy compounds).

When insufficiently purified, or when a small amount of catechol, caffeic acid or protocatechuic acid was added to the purified enzyme, it gave positive reactions with various enzyme indicators. Purification by repeated grindings with acetone removes not only olive oil but also tannins, anthocyanin and simple orthodihydroxy substances. If not sufficiently extracted with acetone, traces of orthodihydroxy substances remain and give a false positive reaction for peroxidase with benzidine or guaiacol and  $\text{H}_2\text{O}_2$ . In our preliminary experiments this fact was not fully appreciated.

Oxidase activity was completely destroyed by incubation of the purified enzyme preparation with trypsin, an indication that the oxidase is proteinaceous in character. A qualitative test on the ground

tissues with the Thunberg technique was negative for oxido-reductase (dehydrogenase). In applying this test, fresh olive pulp in one series, and the partially purified enzyme in another, were mixed with dilute methylene blue and with dextrose and several other substrates in a Thunberg test tube under vacuum. Bleaching of the M.B. would indicate the presence of a dehydrogenase.

### *Quantitative Data*

The effect of the pH value on the purified enzyme, effect of various substances, and temperature, was studied quantitatively by colorimetric measurement, and a few factors only were studied by means of  $O_2$  absorption by the pulp in a Barcroft-Warburg apparatus.

*Preparation of Enzyme.* Fresh pitted or frozen pitted olives were first ground fine in a nut butter blade of a kitchen food grinder. This material was then ground in a mortar with acetone and sand. The mixture was centrifuged and the acetone portion discarded. The sediment was mixed with alcohol, ground and centrifuged. This cycle was repeated a second time. A final treatment with chloroform or ether was given to remove final traces of oil. The finely divided solid residue was air dried and extracted with phosphate buffer at pH 6.7 three times. The enzyme was precipitated by alcohol, filtered and redissolved in phosphate buffer. The resulting solution was nearly water-white and clear. This is essentially the method followed by Hussein and Cruess (4). Unfortunately, this method apparently left a trace of natural catechol-containing substance in the preparation, causing it to react with guaiacol and  $H_2O_2$ , even if no catechol were added. See also the paragraph on qualitative tests.

*Colorimetric Measurement of the Oxidase Activity.* The colorimetric procedure was as follows: 0.5 cc. of the enzyme extract, 0.5 cc. of 2% guaiacol, 0.5 cc. of 0.2 N  $H_2O_2$ , and 2 cc. of phosphate buffer (its pH was varied with condition of test), and  $H_2O$  to make 10 cc., were incubated at 30°C. for one hour. The reaction was then stopped with 2 cc. of glacial acetic acid, and the color formed was measured within 15 minutes with an Evelyn colorimeter in comparison with check measurement on the reagents.

*Observations with Barcroft-Warburg Apparatus.* Using a Barcroft-Warburg type manometric apparatus built in the laboratory, at 30°C., the rate of  $O_2$  absorption by 10 g. of the ground tissue, 20 cc. of phosphate buffer of pH 6.7, and 5 cc. of 0.5% catechol, was measured at regular intervals during a 60 minute period. Blank runs were made with the boiled pulp and reagents. These gave practically no  $O_2$  absorption in 60 minutes, whereas the unheated pulp gave rapid absorption of  $O_2$ .

Olives previously stored several months in brine gave a much less active pulp than did the freshly picked fruit, indicating considerable decrease in enzyme activity during brine storage. Salt was removed by dialysis, as it inhibits oxidase activity.

In one experiment a 0.5% solution of the purified bitter principle from fresh olives was added, instead of 0.5% catechol.  $O_2$  absorption was even more rapid than with an equivalent amount of catechol.

The bitterness yields protocatechuic acid, on alkali hydrolysis. In this case the bitter glucoside was purified by repeated solution in acetone, evaporation of solvent, precipitation with lead acetate from aqueous solution, and treatment of the lead precipitate with  $\text{H}_2\text{S}$ .

These data would tend to substantiate previously mentioned qualitative tests which indicated that the oxidase of the olive is a phenolase, rather than a peroxidase.

*Color Formation vs. Time.* Over a 60 minute period, the amount of color formed by oxidation of guaiacol in the presence of  $\text{H}_2\text{O}_2$  was roughly proportional to the time.

*Effect of Enzyme Concentration.* The amount of color formed per unit of purified enzyme on the dry basis with guaiacol and  $\text{H}_2\text{O}_2$  varied somewhat with the enzyme concentration; the graph showed an optimum range between concentration and color formation. Owing to the relatively low purity of our preparation no attempt was made to express this relationship in absolute values. However, the following data are typical: At maximum concentration of "10," and 60 minutes incubation, the relative color produced was taken as 100. At a dilution  $1/2$  the above, the color produced was 48.2; multiplied by 2 this gives 96.4, comparable to the undiluted at 100. At a dilution of 5:1 it was 14.8;  $5 \times 14.8 = 74.0$ , compared with 100 for the undiluted. With a dilution of 10:1 the color formation was 5.2;  $5.2 \times 10 = 52$ .

*Effect of pH.* Using guaiacol and the purified enzyme, maximum color formation in 60 minutes occurred at approximately pH 5.0 ( $\pm 0.5$  pH unit).

Using the Barcroft-Warburg manometer, and catechol as the substrate, the optimum pH was about 7.5. Hence, it is probable that in these tests the controlling factor was the substrate's pH optimum, rather than that of the enzyme.

TABLE I  
*Effect of pH Value on Olive Oxidase Activity*

pH Value	Per Cent of Maximum Activity
3	56
4	88
5	100
6	90
7	48
8	20
Blank	0

Table I gives typical data for effect of pH value on the formation of color by purified olive oxidase acting on guaiacol and  $\text{H}_2\text{O}_2$ .

*Effect of  $\text{H}_2\text{O}_2$  Concentration.* Several experiments were made to ascertain the optimum  $\text{H}_2\text{O}_2$  concentration. Table II gives typical

TABLE II

*Effect of  $\text{H}_2\text{O}_2$  Concentration on Olive Oxidase Activity with Guaiacol and  $\text{H}_2\text{O}_2$*

Normality $\text{H}_2\text{O}_2$	Per Cent of Maximum Activity
0.00	0
.001	32.5
.002	59.2
.004	74.2
.006	87.0
.008	100.0
.010	92.9
.015	78.5
.020	51.2

data. The optimum concentration appeared to be at about 0.008  $N$ , or about 13.0 mg./100 cc.

At 0.020  $N$   $\text{H}_2\text{O}_2$  there was a marked reduction in activity. Our results are similar to those reported by Cruess and Fong (2) for apricot oxidase.

*Effect of Temperature.* The amount of color found with guaiacol and  $\text{H}_2\text{O}_2$  by the purified enzyme was observed at 5, 10, 13, 20, 26.5, 31.5, 35, 37.5, and 40°C. The maximum was at 31.5°C. At 37.5 and 40°C. it was considerably less. At 5°C. it was only about one-fourth as rapid as at 31.5°C.

Death temperature of the purified enzyme at pH 6.7 was approximately 80°C. for a holding period of 5 minutes at this temperature. Small, thin walled test tubes were used for rapid heating and cooling.

*Effect of NaCN.* Cyanide ion is extremely toxic to the purified enzyme solution, as shown by typical data given in Table III.

Probably the effect is chiefly on the metallic portion of the enzyme molecule (Fe or Cu).

*Effect of KF.* The fluoride ion is also very toxic to the oxidase but less so than the cyanide ion. Thus at 0.005  $N$  KF the activity was only 4.5% of that in the absence of KF, and at 0.0005  $N$  it was 56.8%.

*Effect of NaCl.* Owing probably to an effect on the indicator itself, NaCl exhibited peculiar action. Thus, taking the activity in the

TABLE III  
*Effect of NaCN on Purified Olive Oxidase Activity*

Molal Concentration of NaCN	Per Cent of Maximum Activity
0	100
0.000005	76.0
0.00001	31.8
0.00002	18.5
0.00003	12.8
0.00005	8.7
0.00010	4.1
0.00020	0.0

absence of NaCl as 100, the activities at 0.005 *N* NaCl were 96.4; at 0.10 *N*, 77.5; at 0.20 *N*, 76; and at 0.30 *N*, 96. In other words, there was a minimum at about 0.2 *N*.

*Effect of OH.* As NaOH and KOH are used in the pickling of olives, it seemed of interest to observe the effect of various OH concentrations (using KOH) on the oxidase activity. Table IV gives typical data. The enzyme preparation was subjected to the KOH concentrations noted for a short period, neutralized and then allowed to react with the indicator and H<sub>2</sub>O<sub>2</sub> as previously described.

TABLE IV  
*Effect of KOH on Oxidase Activity*

Normality of KOH	Approximate pH Value	Per Cent Maximum Activity
0.00	6.7	100.0
.01	12.0	93.3
.02	12.3	82.9
.03	12.5	62.4
.04	12.6	0

Evidently our enzyme preparation was destroyed in one hour at or below 0.04 *N* KOH, or about pH 12.6. As the enzyme preparation has some buffering power, the pH will probably vary to some extent with different enzyme preparations.

*Effect of Fe<sup>+++</sup> and Fe<sup>++</sup>.* The addition of a small amount of Fe<sup>+++</sup> (0.001 *N*) increased the enzyme activity 40%. A similar concentration of Fe<sup>++</sup> completely inhibited its activity.

*Effect of Cu<sup>++</sup>.* The addition of Cu<sup>++</sup> to give 0.001 *N* slightly increased the amount of guaiacol oxidized.

*Effect of Oxalate.* Overholser and Cruess, 1923, reported that oxalate inhibits apple oxidase activity in the natural apple tissue. It appeared to have no effect on our purified olive oxidase preparation.

### SUMMARY

1. Qualitative colorimetric tests upon olive tissue and the properly purified oxidase indicate that the oxidase of the olive behaves in a manner characteristic of a phenolase, rather than a peroxidase; *i.e.*, the presence of an orthodihydroxy substance is essential for a positive reaction with certain oxidase indicators.

2. A simple method of preparing an olive phenolase solution free of orthodihydroxy compounds was devised, making use of acetone as the purifying agent.

3. A colorimetric method using guaiacol as the substrate in the presence of  $\text{H}_2\text{O}_2$ , and measuring color by an Evelyn photoelectric colorimeter, proved very satisfactory.

4. The natural bitter principle of the olive (oleuropein) will activate the purified phenolase; *i.e.*, acts as an "oxygenase" (of Onslow). This glucoside is known to be rich in orthodihydroxy groupings.

5. The ground tissue absorbed oxygen quite rapidly in the Barcroft-Warburg apparatus, with or without added catechol, owing to presence of olive bitterness, a catechol-containing substance; further evidence that the oxidase is a phenolase.

6. The oxidation of guaiacol in the presence of  $\text{H}_2\text{O}_2$  catalyzed by the olive oxidase appears to be a first order reaction.

7. With guaiacol as the substrate, the optimum pH value for the purified enzyme was about pH 5; with the ground tissue and catechol as the substrate it was about pH 7.5, indicating that the substrate, rather than the enzyme, may have been the controlling factor in these cases.

8. The optimum  $\text{H}_2\text{O}_2$  concentration was about 10–20 mg./100 cc.

9. The optimum temperature for the purified oxidase acting on guaiacol was about 31°C. Its death temperature at pH 6.7 was about 80°C.

10. The enzyme is extremely sensitive to cyanide ion, being completely inhibited at 0.00020 *M* NaCN. Fluoride ion was also quite toxic, but much less so than CN.

11. 0.04 *N* KOH destroyed the enzyme in one hour or less.



12. Addition of a small amount of  $\text{Fe}^{+++}$  increased the purified enzymes' activity;  $\text{Fe}^{++}$  completely inhibited it.

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# The Role of Pantothenate in Sulfapyridine-Induced Achromotrichia <sup>1</sup>

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Received June 19, 1947

## INTRODUCTION

During the course of studies in this laboratory on the effect of certain aromatic compounds on growth (1), it was found that a synthetic diet low in protein (casein) and containing sulfapyridine alone or together with L-cystine or DL-methionine induces symptoms, chiefly those of pantothenic acid deficiency, which are reversed by supplementation with extra calcium pantothenate. It was thought that further evidence for the participation of this vitamin in the metabolism of sulfapyridine could be adduced by feeding the compound to black rats. In these animals inhibition of pantothenate would be expected to produce an achromotrichia which should be ameliorable by later supplementing the diets with extra pantothenate.

## EXPERIMENTAL

Young black rats were allowed a diet similar in composition to that used by Unna *et al.* (2). The basal ration contained dextrose, 68; casein, 18; hydrogenated cottonseed oil,<sup>2</sup> 8; salt mixture,<sup>3</sup> 4; and cod liver oil, 2 g.-%. Each 100 g. of food contained, in place of an equal amount of dextrose, calcium pantothenate, 1; thiamine chloride, 0.8; riboflavin, 0.8; pyridoxine hydrochloride, 0.8; nicotinic acid amide, 10; and choline chloride, 100 mg. Experimental diet No. 1 was prepared in the same manner, except that the calcium pantothenate was omitted while experimental diet No. 2 carried the calcium pantothenate and in addition 1 g.-% of sulfapyridine. The animals were kept in raised cages with large mesh wire bottoms and allowed food and water *ad libitum*. They were observed for deficiency symptoms, including changes in pigmentation of

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<sup>1</sup> Aided by grants-in-aid from the Nutrition Foundation, Inc.

<sup>2</sup> Crisco.

<sup>3</sup> Osborne and Mendel.

the fur. After 48 days all the diets were supplemented with 10 mg.-% of calcium pantothenate. A total of 43 rats was used.

Sulfapyridine was also incorporated in a diet of natural materials,<sup>4</sup> an experiment which was suggested by that of Wright and Welch (3), who reported no demonstrable effect on the rate of growth of rats in similar experiments in which succinylsulfathiazole was incorporated. In our experiments the drug was simply mixed with the powdered food at levels of 1, 5 and 10% and the animals were observed for deficiency symptoms and changes in the growth rate.

## RESULTS

The animals receiving the pantothenate-free diet grew at a slower rate as compared with those fed the basal diet and the growth was retarded still more in the animals receiving experimental diet No. 2 containing sulfapyridine and calcium pantothenate. Definite signs of depigmentation appeared in 3-4 weeks. The graying was quite conspicuous in the rats receiving the experimental diets. In the controls receiving the basal diet a slight amount of graying was also evident as



FIG. 1. This rat received the experimental diet containing sulfapyridine and pantothenate. The picture was taken at the end of the 48-day period.

was to be expected from the finding of Unna *et al.* (2) that 100  $\gamma$  daily of pantothenate is required to prevent achromotrichia. Since the basal animals ingested on the average less than 10 g. of food per day they were getting somewhat less than the prophylactic amount of the

<sup>4</sup> Purina Brand.

vitamin. The graying seen in the animals receiving the pantothenate-free diet was most marked in a symmetrical pattern along the sides of some of the animals while in others it included the entire back. The depigmentation in the animals receiving the sulfapyridine (see Fig. 1)



FIG. 2. This rat received the basal diet. The picture was taken at the end of the 48-day period.

spread over the entire back. In a large percentage of the animals receiving the sulfapyridine alopecia developed, sometimes over part of the back beginning at the base of the tail, sometimes over the neck and shoulders, and occasionally on the sides of the abdomen. The hair became rough and frequently scant in the areas where the graying occurred and there was also observed a brownish exfoliative dermatitis usually beginning at the base of the tail. The animals on both experimental diets showed coproporphyrin deposits on the noses, wrists and whiskers. In some animals receiving the sulfapyridine diet the so-called blood-caked whiskers was extremely marked. Occasional animals on this diet also exhibited a hematuria.

After 48 days the graying was quite marked and all the rations, including the basal diet, were supplemented with 10 mg.-% of calcium pantothenate. After 20 days the fur of the controls had become quite black and that of the sulfapyridine rats was beginning to show some pigmentation. After 28 days on the high pantothenate diet the pigmentation was nearly complete in all the animals except for a few scattered gray hairs which showed even in the controls and the experiment was discontinued.

Supplementation of the basal diet did not result in any stimulation

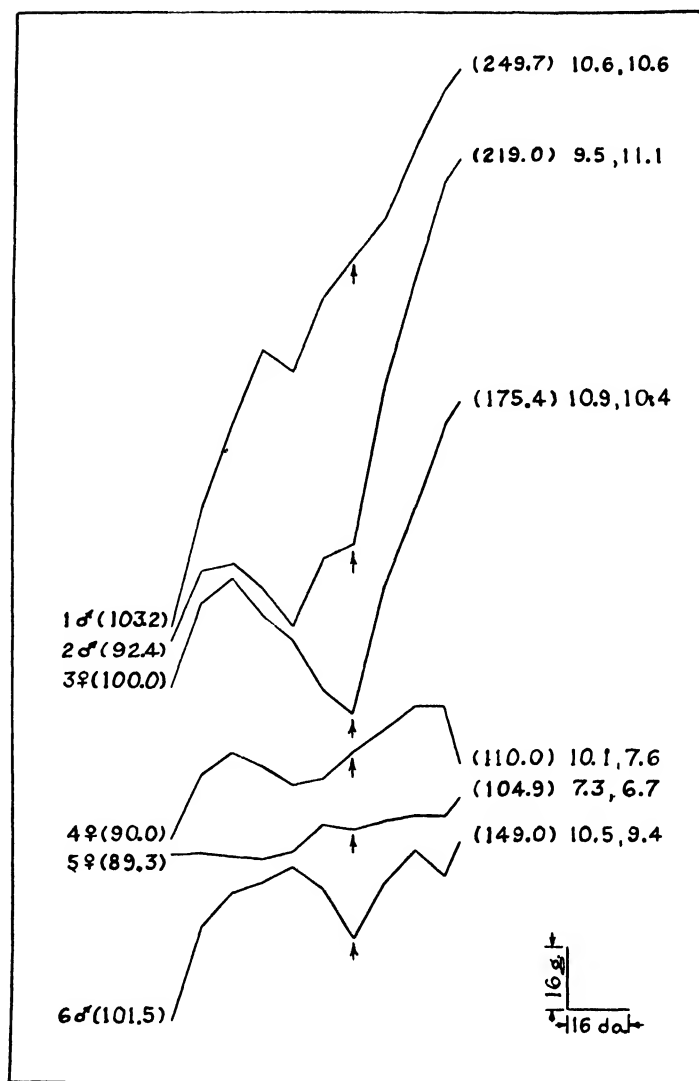


FIG. 3. The effect of sulfapyridine on the growth of young black rats. Rat number 1 received the basal diet. Rats 2 and 3 received experimental diet No. 1 which was pantothenate free. Rats 4, 5 and 6 received experimental diet No. 2 containing pantothenate and 1% sulfapyridine. At the end of 48 days 10 mg.-% of calcium pantothenate was added to all of the diets. The initial and final weights are given in parentheses. Average daily food consumption for both periods is shown at the ends of the curves.

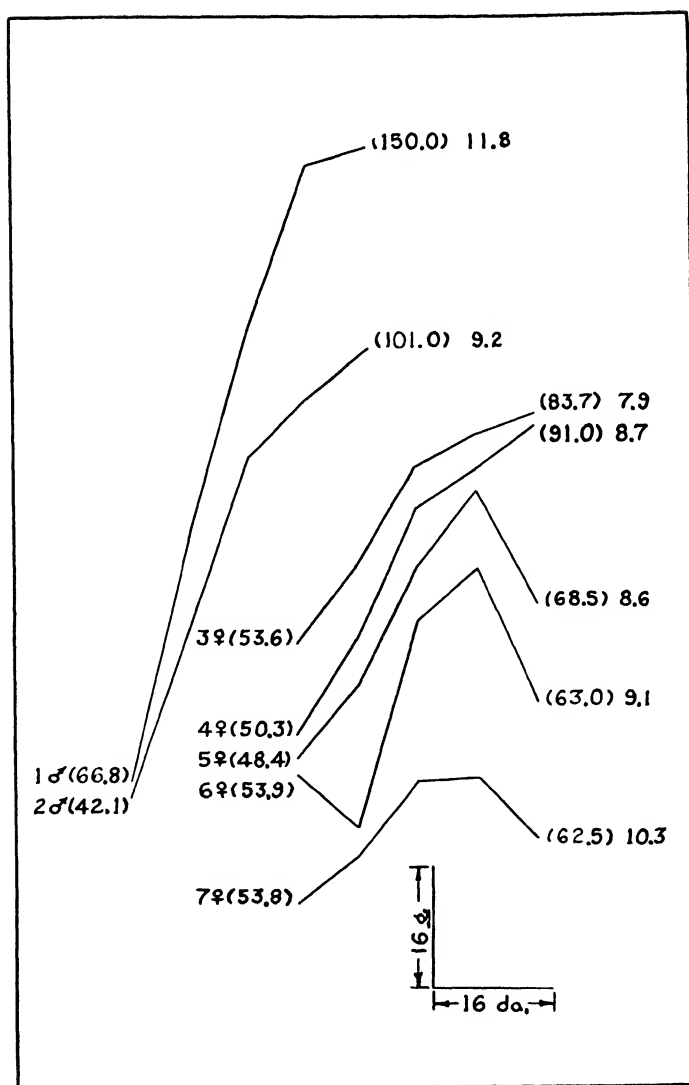


FIG. 4. The effect of sulfapyridine on the growth of young black rats subsisting on a diet of natural foodstuffs.<sup>4</sup> Rats 1 and 2 received a basal diet consisting of the powdered foodstuff. Sulfapyridine was incorporated in the diets of rats 3 and 4 at a level of 1%, in the diet of rat number 5 at a level of 5% and in the diets of rats 6 and 7 at a level of 10%. The initial and final weights are given in parentheses. The average daily food consumptions are given at the ends of the curves.

of growth but, as was to be expected, marked increase in the rate of growth occurred when the pantothenate was added to experimental diet No. 1. Extra pantothenate added to the sulfapyridine diet caused the disappearance of the symptoms of pantothenate deficiency but was without effect in stimulating growth of the animals.

When sulfapyridine was included in the diet of natural foodstuffs at levels of 1, 5 and 10% there was marked retardation of growth at all three levels of the drug, and thinning of the hair, which progressed to completely bald areas on the backs of some of the animals. Coproporphyrin deposition was extensive and the brownish exfoliative dermatitis mentioned above was also present.

### DISCUSSION

In line with earlier findings (1), inclusion of sulfapyridine in the diets of young rats again produced symptoms of pantothenic acid deficiency, including roughening of the fur, coproporphyrin deposits on the noses, wrists and whiskers, and retardation of growth. In addition, in the present experiments with black rats achromotrichia has been produced. An additional symptom of pantothenate deficiency, hemorrhagic necrosis of the adrenal glands, was reported earlier (1) for sulfapyridine. These symptoms have been repeatedly reported for pantothenate deficiency and their appearance following administration of sulfapyridine strengthens the concept that this substance interferes in some way with pantothenate metabolism. This conclusion is further strengthened by the fact that feeding a comparatively large supplement of the vitamin reverses all of the symptoms observed in these experiments except one, the retardation of growth. Since extra pantothenate does not relieve the growth inhibition, it is evident, as we pointed out previously (1), that the deficiency induced by feeding sulfapyridine is multiple in nature.

Incorporation of sulfapyridine at various levels in a complete diet of natural foodstuffs produced retardation in growth, and deficiency symptoms which were in striking contrast to the observations of Wright and Welch (3) with succinylsulfathiazole. These authors found no demonstrable effects on the rate of growth of rats in comparison with the growth of animals on the same diet without drugs. Their animals appeared to be normal in every way and no evidence of pathological changes was demonstrable histologically. They found, however,

that much smaller amounts of succinylsulfathiazole (1 or 2%) were effective in producing marked inhibition of growth when added to purified rations otherwise adequate in their content of the hitherto recognized essential dietary factors. Apparently, nutritive factors required for growth stimulation of animals receiving sulfapyridine are not the same as those needed for animals fed succinylsulfathiazole.

#### ACKNOWLEDGMENT

The authors gratefully acknowledge the technical assistance of Mr. Eusebius M. Barron in the conduct of these experiments.

#### SUMMARY

Sulfapyridine fed at a level of 1% in a synthetic diet given black rats induces achromotrichia and other symptoms of pantothenic acid deficiency.

The achromotrichia, as well as the other symptoms of pantothenic acid deficiency, are relieved by supplementation of the diets carrying the drug with extra pantothenate, although the supplement is without effect on the growth retardation produced by feeding sulfapyridine.

Sulfapyridine inhibition of pantothenate appears to be a factor in the deficiency syndrome produced by administration of this drug.

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# On the Participation of Carbon Dioxide in the Photosynthetic Activity of Illuminated Chloroplast Suspensions

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Received June 28, 1947

## INTRODUCTION

Among the earliest observations of the evolution of oxygen by isolated chloroplasts were those of Haberlandt (1887) and Ewart (1896) using the oxygen sensitivity of chemiluminescent bacteria as a test reaction. Hill (1937) demonstrated that illuminated chloroplast suspensions were able to produce relatively much larger amounts of oxygen when supplied with suitable oxidants. Warburg (1944) and French and Holt (1946) have shown that a variety of substances foreign to the green cell may serve as oxidants for the photochemical oxidation of water to oxygen. Such reactions proceed without carbon dioxide, demonstrating that the so-called "Hill reaction" is concerned only with the photochemical and oxygen-liberating portions of the photosynthetic mechanism. If any carbon dioxide fixation occurs in chloroplast suspensions, as it does in intact photosynthesizing cells, then it must be at a much reduced rate not detectable by macro-methods.

Franck (1945) studied the oxygen production of illuminated tobacco chloroplasts with an apparatus capable of measuring oxygen partial pressures as low as  $10^{-6}$  mm. Hg. He and his co-workers observed that, at the beginning of an illumination, a burst of oxygen was produced. The rate of evolution declined rapidly and, after several minutes, a nearly constant but slowly declining rate was maintained for many minutes (up to one hour or more). The total amount of oxygen produced was so small that it would not be measurable except by such a micromethod. Upon investigating the influence of carbon dioxide on this oxygen production, Franck reported that the

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final, nearly steady rate was greater in the presence of carbon dioxide than in its absence. The results were explained as follows: The initial high rate of oxygen evolution was accounted for as a release of oxygen due to further reduction of some intermediate (left over from previous photosynthesis or produced by metabolism in the dark). The subsequent steady rate of oxygen production was attributed to photosynthetic reduction of carbon dioxide taken up by the chloroplasts at a very low rate. As the rate limiting factor, there was postulated a deficiency of the mechanism of non-photochemical fixation of carbon dioxide. That this mechanism should be deficient in isolated chloroplasts as compared with intact cells is consistent with the findings of Frenkel (1941) which demonstrated that the dark fixation of carbon dioxide by *Nilella* cells is a reaction occurring chiefly outside the chloroplasts.

By employing  $C^{14}$  as a tracer, we have been able to test whether or not carbon dioxide actually is reduced or fixed by isolated chloroplasts as must be expected on the basis of Franck's interpretation of the increased oxygen production which results from the presence of this gas. Stated briefly, our results indicate no significant fixation or reduction of carbon dioxide by chloroplasts.

The total carbon dioxide uptake to be expected in a chloroplast experiment could be calculated from observed rates of oxygen evolution in smaller scale tests with chloroplast suspensions. In an experiment of feasible dimensions this proved to be of the order of one microliter ( $\mu$ l) of carbon dioxide (about  $2 \times 10^{-6}$  g. carbon). Using an external counter with a thin mica window, it is possible to measure the radioactivity of a sample containing as little as  $1 \times 10^{-9}$  g.  $C^{14}$  or less. The tracer, therefore, could be used in moderate dilution. However, since the measurement is not very far in excess of the limit of sensitivity of the tracer technique as we have applied it, we shall report our results in sufficient detail to demonstrate that our negative findings were not due to insensitivity of the experimental method.

## MATERIALS AND METHODS

Chloroplasts were prepared from healthy young tobacco leaves from greenhouse-grown plants in a standardized manner essentially similar to the method employed in the previous experiments in this laboratory.

Fifteen or twenty g. of fresh leaf material were disintegrated in one minute in a Waring Blendor with 100 ml. of 0.25 *M* sucrose and 0.05 *M*  $K_2HPO_4$ . The macerated tissue was filtered through cotton and centrifuged in 50 ml. cups in an International clinical model centrifuge for 3 minutes at low speed to eliminate the larger particles. The supernatant fluid was centrifuged 5 minutes at high speed. This left most of the grana and smaller starch grains in the suspension which was discarded. The chloroplasts were washed in 0.30 *M* sucrose solution and were suspended in about 10 ml. of

0.30 *M* sucrose and 0.05 *M* phosphate buffer of either pH 5.9 or pH 8.4. The entire preparation was performed in very dim light in a refrigerated room at  $5^{\circ} \pm 1^{\circ}\text{C}$ . Upon maceration of the leaf tissue, the chloroplasts were never exposed to light until the beginning of the experimental illumination.

The tracer carbon was obtained through the U. S. Atomic Energy Commission as enriched barium carbonate. This was converted to  $\text{Na}_2\text{CO}_3$  and used as a solution containing  $940\ \mu\text{l CO}_2/\text{ml}$ . and, in terms of radioactivity, each ml. of solution contained  $2.3 \times 10^6$  counts/minute (c/m) observable by the counting method we used.

Each sample was counted either directly as a solid spread over a known area or, after wet combustion and conversion of the carbon dioxide to carbonate, as solid barium carbonate in the same way. Appropriate corrections were made for background count and sample thickness (self-absorption) in the conventional manner.

The measurements of radioactivity thus were all relative, since no corrections were made for the radiation absorbed by the mica window or for the sample-counter geometry. Since samples were always counted with the same tube in a holder which insured their identical orientations, these latter factors were constant.

## EXPERIMENTAL RESULTS

### (a) *Aerobic Conditions*

Because it was technically easier, a preliminary experiment was performed at  $20^{\circ}\text{C}$ . in open vessels in a darkened room. Two suspensions containing about 10 mg. (dry wt.) of freshly isolated chloroplasts were exposed to 0.5 ml. of the enriched sodium carbonate solution for 5 minutes. One sample was illuminated at what would be about the saturation intensity for photosynthesis in an intact leaf. The other remained dark. Boiling 1 *N* acetic acid was added to both samples at the end of 5 minutes to terminate all enzyme reactions. The suspensions were heated on a steam bath for 15 minutes and the acid extract separated from the residue by centrifugation. The extract was evaporated

TABLE I  
*Radioactivity Measurements on Extracts and Chloroplast  
Residues in Aerobic Experiment*

Available radioactivity,  $11.5 \times 10^6$  c/m. Temperature,  $20^{\circ}\text{C}$ . Time, 5 min. Tabular values are observed counts/minute. Standard errors of measurements are expressed as per cents and calculated as  $100/\sqrt{n}$  where  $n$  = total number of counts observed.

Illuminated chloroplasts	{ Acetic acid extract	$60.0 \pm 2\%$
	{ Residue	$56.6 \pm 2\%$
Unilluminated chloroplasts	{ Acetic acid extract	$60.7 \pm 2\%$
	{ Residue	$58.7 \pm 2\%$
Background		$56.5 \pm 2\%$

to dryness and both extracts and chloroplast residues were counted as dry solids. As the results in Table I show, not only are the activities of all samples of dubious significance compared with the background count but the difference of the value for illuminated minus that for unilluminated suspension is negative, both in the case of the extracts and in the case of the residues. Since all differences from background cannot be considered significant, corrections for sample self-absorption (or for activity due to  $K^{42}$  which was present in the potassium of the buffer) would be meaningless.

(b) *Anaerobic Conditions*

Since Franck's original observations were made at 0°C. under conditions of extreme anaerobiosis, we attempted to reproduce these experimental conditions by working entirely in a refrigerated room in very dim light prior to mixing the suspensions with enriched  $Na_2CO_3$  solution. Vessels containing chloroplast suspensions, each with the radioactive carbonate solution in a side arm, were flushed out with tank nitrogen and, before tipping in the carbonate, the vessels were brought to an exhaust hood in a Dewar flask protected from light. The suspension to be illuminated was contained in an ice bath so that both

TABLE II  
*Radioactivity Measurements on Extracts and Chloroplast  
Residues in Anaerobic Experiment*

Available radioactivity,  $11.5 \times 10^5$  c/m. Temperature 0°-5°C. Time, 5 min. Tabular values are counts/minute. Standard errors of measurements are expressed as per cents and calculated as  $100/\sqrt{n}$  where  $n$  = total number of counts observed.

	Fraction	Observed activity	Corrected <sup>a</sup> activity
Illuminated chloroplasts	Acid extract	$57.6 \pm 2\%$	$165 \pm 93\%$
	Residue	$58.8 \pm 2\%$	$11 \pm 54\%$
Unilluminated chloroplasts	Acid extract	$59.1 \pm 2\%$	$297 \pm 52\%$
	Residue	$60.2 \pm 2\%$	$15 \pm 39\%$
Background		$56.1 \pm 2\%$	

<sup>a</sup> Corrections made for sample aliquot, self-absorption, and background. The large errors of the values for extracts are due to the large amount of sucrose present in the experimental suspensions which made the observed count very low.

"light" and "dark" chloroplast samples were at nearly 0°C. until the boiling acetic acid was added. In these experiments about 25 mg. of chloroplasts were employed in both "dark" and "light" experiments. Table II shows that practically no radioactivity was fixed either in the dark or in the light. Significant reduction would be shown by a positive light-dark difference. Here, as in Table I, this difference is in each case negative and of no significance.

From earlier work of Ruben *et al.* (1940) on photosynthesis with  $C^{11}$ , as well as from more extensive observations in this laboratory with  $C^{14}$ , it has become apparent that the so-called "first product" of photosynthesis is a remarkably stable substance which can be extracted quantitatively with dilute acid. Therefore, the acid extract from illuminated chloroplasts is deserving of our special attention. To avoid any necessity of correcting for potassium activity (which could amount to perhaps 3 c/m in this case), and especially to insure homogeneity in the sample counted, the acid extract was burned by wet combustion and counted in the form of  $BaCO_3$  as mentioned above. In this case  $2.9 \pm 24\%$  c/m over the background were observed and, correcting for self-absorption, the sample contained  $149 \pm 24\%$  c/m which agrees satisfactorily with the upper value in Table II.

### DISCUSSION

The significance of the above results with  $C^{14}$  must be evaluated in terms of the fixation to be expected. Using the type of apparatus described by Pollack, Pringsheim and Terwoord (1944), oxygen production *vs.* time curves were obtained<sup>2</sup> which were similar to those reported by Franck. Our chloroplasts were about 5 or 10 times as active as were those used in previous experiments in this laboratory. Correcting for the quantity of chloroplasts used in the  $C^{14}$  experiments (which were on a 50 times larger scale than the tests in which oxygen production was measured), we were able to estimate the probable oxygen production during the 5 minutes of illumination in our  $C^{14}$  experiments. Reasoning conservatively that any possible carbon dioxide fixation should correspond to the relatively low, nearly steady rate of oxygen evolution by isolated chloroplasts rather than to the total amount of oxygen included in the initial burst, we should expect about 0.5 to

<sup>2</sup> Mr. George Zimmerman performed the measurements of oxygen production by illuminated chloroplast suspensions.

1.0  $\mu$ l of carbon dioxide to be reduced during 5 minutes of illumination. In our experiments this would have corresponded to between 1150 and 2300 c/m, values which are very significantly greater than any of the activities we observed. We must conclude that the effect of carbon dioxide in augmenting oxygen evolution by illuminated chloroplasts is not the result of additional carbon dioxide being taken up by the chloroplasts once they are isolated from the plant cell.

Further studies of factors influencing oxygen production by illuminated chloroplast suspensions have been carried out in an effort to understand the processes involved. These experiments are not yet completed and their results, as well as the significance of the oxygen production by isolated chloroplasts in relation to the mechanism of photosynthesis, will be discussed at a later time.

### SUMMARY

In earlier studies of the oxygen production by illuminated chloroplast suspensions, it was reported that the presence of carbon dioxide enhanced the rate of oxygen evolution. Using  $C^{14}$  as a tracer, it was found that significant amounts of carbon dioxide were neither reduced nor fixed by isolated chloroplasts. Therefore, increase in oxygen production in the presence of carbon dioxide cannot be interpreted as a complete cycle of photosynthesis in chloroplasts.

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# Crystalline Pepsin-Resistant Protein from Skeletal Muscle

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Received July 9, 1947

## INTRODUCTION

In a recent publication (1), a description was given of a crystalline substance extracted from commercial pepsin, which was of very high molecular weight, had some of the characters of a protein or large polypeptide, and was not susceptible to peptic hydrolysis; the term *peptophan* was proposed for this substance. Attempts to extract similar material from peptic digests of various tissues have resulted in the isolation from skeletal muscle of a crystalline, protein-like substance also characterized by its high resistance to peptic hydrolysis. This substance, which will be referred to as *peptomyosin*, is described in the present paper. The details which follow refer to the preparation of peptomyosin from beef muscle. The preparation and properties of horse peptomyosin, which differ slightly, are described in a later section.

## EXPERIMENTAL

### *Isolation of Beef Peptomyosin*

In a Waring Blendor were placed 250 g. of chopped, defatted, beef muscle and 500 ml. of distilled water. The material was usually a few weeks old and preserved in the frozen state. The mixture was ground for from 5 to 10 minutes, and was then transferred to a beaker and stirred mechanically with a metal stirrer provided with sharp blades. A large amount of fibrin-like material collected on the stirrer, from which it was removed every few minutes until the suspension was free from it.<sup>1</sup> The suspension was then made up to 1 liter with water, distributed into four 250-ml. centrifuge bottles, and centrifuged for 30 minutes. The sediment was stirred up with water (a small mechanical stirrer was helpful) and recentrifuged. This operation was repeated 4

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<sup>1</sup> With fresh muscle, the pH during this operation is 5.7, which is optimum for the removal of fibrin. When stored meat is used, the pH remains higher and should be adjusted to about 5.7 with HCl.



times until the supernatant, at first deep red and later less and less colored, at last turned whitish and opalescent. The sediment was then suspended in 600 ml. of water, acidified to pH 1.5 with about 70 ml. of  $N$  HCl, and 0.5 g. of pepsin (Parke, Davis and Company, 1:10,000) was added. (The pepsin was first dissolved in a little water and added at the same time as the acid, to avoid gelling of the suspension.) This was left overnight at  $38^{\circ}\text{C}$ ., neutralized to pH 4.6 with about 55 ml. of  $N$  NaOH, heated to  $50^{\circ}\text{C}$ . in a water bath, and cooled. The precipitate that formed was filtered off on coarse paper (Eaton and Dikeman No. 615). The first portions were refiltered when necessary. To the clear filtrate was added 0.8 volume of saturated ammonium sulfate solution. After an hour, the material was centrifuged down, dissolved in 200 ml. of 0.1  $N$  acetate buffer pH 5.0, reprecipitated, suspended in a little water, and dialyzed in a cellophane bag against running tap water.

### *Crystallization*

The cellophane bag was placed in 0.2  $M$   $\text{Na}_2\text{HPO}_4$  until the material dissolved to form an opalescent solution. The bag was then immersed in several liters of 0.01  $M$   $\text{NaH}_2\text{PO}_4$  or  $\text{KH}_2\text{PO}_4$ , containing 'Merthiolate,' 1:20,000, and allowed to stand at room temperature, with occasional stirring, for several days. The crystals were centrifuged down, washed in 0.01  $M$   $\text{KH}_2\text{PO}_4$ , dissolved with the help of  $\text{Na}_2\text{HPO}_4$ , and the material was recrystallized by the same procedure. The suspension of crystals was later dialyzed against water to remove the phosphate. Optimum concentration for crystallization appeared to be from 1 to 3%. The yield of crystals was high, but little material remaining in the mother liquor. The overall yield was about 1 g. of twice-crystallized material from 250 g. of muscle.

### *Physical Properties*

Peptomyosin crystals, as obtained by the above method, were small, flat, occasionally spindle-shaped needles, often packed in clusters (Fig. 1), and formed a suspension of milky white appearance. They dissolved rapidly to a colorless, opalescent solution upon addition of enough HCl to lower the pH to 4, or enough NaOH to raise it above 6. Between pH 4 and 6 they dissolved only in the presence of enough salt. For example, at pH 5.6, the crystals dissolved if the NaCl concentration was  $M/8$  or greater, not if it was  $M/16$  or less. With  $\text{CaCl}_2$ , the limiting molarity was between  $M/32$  and  $M/64$ . The results were the same at  $100^{\circ}\text{C}$ . as at room temperature, except that at  $100^{\circ}\text{C}$ . the undissolved crystals lost their shape. In other words, peptomyosin was not precipitated by boiling, even near its point of minimum solubility, provided enough salt was present.

Peptomyosin precipitated upon addition of trichloroacetic acid, but the precipitate was bulky and gelatinous, and did not resemble that formed by ordinary proteins. Upon heating, the precipitate aggregated

in sticky masses which clung to the surface of the solution, but did not appear to dissolve appreciably. Even the precipitate that formed upon addition of ammonium sulfate had a slightly gelatinous appearance and did not pack well during centrifugation unless allowed to form undisturbed.



FIG. 1. Twice-crystallized beef peptomyosin. ( $\times 600$ )

Finally, in spite of its crystalline character, beef (but not horse) peptomyosin easily formed extremely viscous solutions when the medium was salt-free and the pH not far from that of lowest solubility (about 5.0). This could be best demonstrated by adding to a salt-free suspension of crystals just enough HCl to dissolve (pH 4.0–4.5). Upon standing in the cold room such solutions would occasionally form a gel.

For this reason, the procedure described above for crystallization was successful only at room temperature.

By the author's method (2) beef peptomyosin solutions yielded stable reproducible osmotic pressures, from which an average molecular weight of 91,000 was derived (Table I).

TABLE I

*Molecular Weight of Beef Peptomyosin from Osmotic Pressure Measurements*

Salt solution	pH	Peptomyosin concentration	Calculated molecular weight
		<i>per cent</i>	
0.1 N NaCl, 0.1 N acetate buffer	3.7	0.71	86,000
0.1 N NaCl, 0.1 N acetate buffer	3.7	0.71	92,000
0.1 M phosphate buffer	7.2	1.70	94,000
0.1 M phosphate buffer	7.2	0.17	93,000
Average			91,000

### *Chemical Properties*

Some of the chemical properties are given in Table II. The nitrogen and carbon percentages are those of a protein. The nitroprusside test for SH groups was but weakly positive even when the material had previously been heated with KCN. The Molisch test was weakly positive and indicated only a low percentage of carbohydrate.

TABLE II

*Chemical Properties of Beef Peptomyosin*

Nitrogen (Kjeldahl), per cent	16.2
Carbon (Van Slyke and Folch), per cent	50.2
Phosphorus (phosphomolybdate)	—
SH (nitroprusside)	±
SH (NaOH and lead acetate)	+
Carbohydrate (Molisch)	+
Tyrosine (Folin)	+
Tryptophan (Hopkins-Cole)	—
Biuret	+

Peptomyosin, as the procedure for its preparation shows, proved extremely resistant to hydrolysis by pepsin. A 3.4% solution of twice-crystallized material was acidified to pH 1.5 with HCl, pepsin was added to the extent of 1/100 the amount of substrate, and the solution was left 20 hours at 38°C. The pH being still 1.5, it was brought back to

about 5 with NaOH, the material was redissolved with  $\text{Na}_2\text{HPO}_4$  and dialyzed against 0.01 *N*  $\text{KH}_2\text{PO}_4$ . Crystallization was as satisfactory as in the original procedure, and there was no apparent loss. Peptomyosin was, however, rapidly destroyed by commercial trypsin. When it was mixed with about 1/50 its weight of Pfanstiehl trypsin, 1:110, and the solution adjusted to pH 8.5 with NaOH, the pH dropped to 7.2 in 2 hours at room temperature and to 6.5 in 20 hours, and the solution at this stage no longer contained more than a trace of material precipitable by 45% saturation with ammonium sulfate.

### *Serologic Properties*

In an attempt to determine whether the material was antigenic, 3 rabbits were given, 12 times in the course of 3 weeks, 0.1 mg. of peptomyosin by the intravenous route. The sera thus prepared gave no precipitin reaction, even by the sensitive so-called "ring" test. This suggested that peptomyosin was not a strong antigen.

### *Preparation and Properties of Horse Peptomyosin*

The initial steps in the preparation of the crude material were exactly as described above; the method for crystallization was slightly different, *viz.*, the suspension obtained after dialysis against tap water was made 0.1 *N* in NaCl and the pH adjusted to 7.5 with NaOH. It was then heated to 50°C. to dissolve, and a small insoluble residue removed by centrifugation. Finally, it was dialyzed in the cold against a buffer that was 0.01 *N* in  $\text{KH}_2\text{PO}_4$  and 0.05 *N* in NaCl. With lower salt concentrations only an amorphous flocculent precipitate appeared, while with higher concentrations spheroids were produced. The yield of crystals was small, only about 20% of the material present, or 0.2 g. from 250 g. of muscle. Upon second crystallization, almost all the material was recovered in crystalline form. The crystals were small irregular needles, usually grouped in the shape of whisk brooms. They were fragile and coalesced rapidly during microscopic examination, but kept their shape better if suspended in distilled water.<sup>2</sup>

The solutions of horse peptomyosin were darker than beef peptomyosin, less viscous, and never gelled, even after prolonged storage in the cold. The behavior toward trichloroacetic acid was the same. The tests were positive for tyrosine-tryptophan (Folin), tryptophan (Hopkins-Cole), and SH groups (NaOH-lead acetate). There was but little carbohydrate (Molisch). Under the same conditions, beef peptomyosin gave a negative test with the Hopkins-Cole reagent, but the difference may not be significant.

The substance gave stable osmotic pressures in 0.06 *M* phosphate buffer, pH 6.9. On the assumption that it contained as much nitrogen as the other (16.2%), the calculated molecular weight was 75,000.

<sup>2</sup> Crystallization can also be brought about by dialyzing a neutral solution of the protein against distilled water for one or two weeks at 5°C.

## DISCUSSION

Peptomyosin appears to have enough physical and chemical characters in common with proteins to warrant including it in the protein class. However, in its resistance to peptic hydrolysis, it deviates from the behavior of ordinary proteins and resembles the peptophan isolated from commercial pepsin (1). The latter was further distinguished by its resistance to tryptic hydrolysis as well, and its ability to form, in boiling salt-free water, concentrated solutions from which it crystallized upon cooling. Both substances have a molecular weight of the order of magnitude found for proteins, and both have minimum solubility at about pH 5.0. On the other hand, once-crystallized peptophan contains neither sulfur nor carbohydrate, whereas twice-crystallized peptomyosin contains both.

These findings raise several questions, namely: whether peptomyosin exists as such in the intact muscle, or is an artifact of preparation; what position the substance occupies in relation to the more or less well-defined protein fractions already isolated from muscle; and, what is its physiologic function.

Whether peptomyosin exists as such in the native muscle, or whether it is an artificial product of peptic hydrolysis is a point on which speculation is of necessity limited by our still near-complete ignorance of the mode of peptic action. It may be remarked, however, that the ability of pepsin to synthesize compounds of high molecular weight is still undemonstrated (3), and that any such compound might be expected to be formed as the result of some unstable equilibrium rather than as an end-product of considerable stability. Peptomyosin was recovered from digests which had been left 3 days at 38°C. as well as from material digested less than 24 hours, and, as shown above, the twice-crystallized material proved extremely resistant to redigestion. Furthermore, in a study of peptic hydrolysis of comparatively pure proteins (such as egg albumin or serum pseudoglobulin) it has been the writer's experience that the isolation of intermediate split products possessing reproducible properties and some appearance of homogeneity is an extremely difficult proposition. These considerations would support the view that peptomyosin exists as such in the intact muscle.

Concerning the second question, the evidence thus far does not suggest that peptomyosin is identical with any of the fractions of muscle described in the literature. The problem is complicated by the fact that most of the information available deals with rabbit and not

with beef muscle, and that the starting material in the present case is finely ground (in the Waring Blendor) by a method not used to our knowledge by other investigators. That this material is different is shown by the appearance of a large amount (about 6 g. dry weight for each 250 g. of wet muscle) of a substance similar to blood fibrin which is not mentioned by other workers. During this extraction the pH of the mixture is about 6, and only an estimated 2-3 g. of protein go into solution. Assuming that 250 g. of muscle contain 50 g. of protein, this indicates that about 4/5 of the material remains for hydrolysis, presumably containing all the myosin, and some other fractions as well.

Some of the properties of the main fractions of muscle may be pointed out here to distinguish them from peptomyosin. For example, the peculiar solubility of myosin in distilled water (4, 5) and in the presence of salts is not duplicated by peptomyosin, and the only point of resemblance would be the latter's ability (when extracted from beef muscle) to form viscous solutions and gel-like precipitates, indicating that the substance may exist in fibrous form. Nevertheless, the ease with which stable osmotic pressures were obtained shows that, in the presence of salts, peptomyosin behaves like any other globular protein. Similarly, actin (5) shows a distinctive behavior in the presence of salts; it is furthermore rapidly destroyed, according to Szent-Györgyi, even at 0°C., when the pH is not at neutrality. The crystalline myosins A and B have the solubility of albumins; myosin B is denatured by heating to 50°C. or acidifying to pH 5 (6). Myoalbumin has an isoelectric point at 3.0-3.5, and both myoalbumin and globulin X are rapidly denatured by acid (7). An unusual instance is offered by myokinase, the enzymic activity of which resists boiling in 0.1 N HCl; however, "myokinase is digested by commercial pepsin, and the activity completely abolished" (8). Of the three enzymes recently isolated from muscle in crystalline form (9), none appears to bear any resemblance to peptomyosin. In general, the instability of the proteins just reviewed (excepting myokinase) stands in marked contrast to the rugged constitution of peptomyosin. The fact that rabbit muscle freed from blood contains at least 11 main components distinguishable by electrophoresis (10), which have not yet been identified with isolated fractions, illustrates the complexity of the composition of muscle.<sup>3</sup> Even crystal-

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<sup>3</sup> To this list should be added tropomyosin, recently isolated by K. Bailey [*Nature* 157, 368 (1946)], which appears to come from the muscle stroma and is unusually resistant to denaturing procedures. The method of extraction and the physical properties do not suggest that it is identical with peptomyosin.

line myosin, formerly considered to be a single entity, is now regarded as a complex, and the existence of stable active components, called "protins," for which myosin acts as a carrier, has recently been postulated (11). It is conceivable that the substance here described may represent one of these protins.

As to the role played by peptomyosin, no suggestion is offered at present. It may be remarked, however, that resistance to peptic hydrolysis is not *per se* a sufficient reason for ruling out the assumption that the substance possesses a specific enzymic function in muscle physiology.

### SUMMARY

A substance having the general properties of a protein has been isolated from peptic digests of beef skeletal muscle.

It is extremely resistant to peptic hydrolysis, is insoluble in salt-free solution at pH 5, and is easily obtained in crystalline form.

The molecular weight (from osmotic pressure determinations) is 91,000.

The substance represents at least 2% of the total proteins and does not appear to be identical with any of the known fractions of muscle. Its physiologic role is unknown.

A similar substance has been extracted from horse skeletal muscle.

The term *peptomyosin* is proposed for this substance.

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# The Effect of Repletion on the Plasma Proteins in the Dog Measured by Electrophoretic Analysis<sup>1</sup>

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Received July 16, 1947

## INTRODUCTION

Whipple (1), and co-workers, determined the restoration of plasma proteins by measuring the amount and kind of protein which is removed daily through plasmapheresis to maintain a dog receiving a test protein in a "standardized" hypoproteinemic condition. They reported that feeding bovine serum protein (2) leads to the restoration of more albumin than globulin, casein (2) of equal amounts of albumin and globulin, and lactalbumin (3) of more globulin than albumin. Melnick, Cowgill and Burack (4, 5), using a similar technique, found that bovine serum protein, casein, and lactalbumin were equivalent in restoring total plasma proteins in hypoproteinemic dogs, but the kind of protein restored was not determined.

Weech and Goettsch (6, 7) depleted dogs by feeding a low-protein diet for 3 weeks and then measured albumin restoration during a 1-week protein feeding period. They reported that bovine serum protein was more efficient than casein in restoring plasma albumin. Cox and Mueller (8), on the other hand, using the Weech technique, observed that enzymatic hydrolyzates of bovine serum protein, of casein and of lactalbumin were equally effective in restoring plasma albumin.

After depleting dogs by a combination of plasmapheresis and protein-free feeding, Seeley (9) measured the increases in plasma albumin and globulin during and following a 5 day period of feeding test proteins to the hypoproteinemic dogs. He found that casein and bovine serum protein were equally effective in restoring total plasma protein, but the amount of restoration was directly dependent upon the degree of positive nitrogen balance produced. Bovine serum protein led to the restoration of plasma albumin only, while casein feeding restored both albumin and globulin.

Using a similar technique, Allison, Anderson and Seeley (10) measured increases in total circulating protein, albumin, and globulin, over a period of thirty days of repletion, and found that feeding an enzymatic hydrolyzate of casein resulted in restoration of both albumin and globulin.

Chow *et al.* (11) studied the effect of plasma protein depletion in dogs by analyzing the plasma both chemically and electrophoretically.

<sup>1</sup> The authors are indebted to Mrs. Shirley DeBiase for her technical assistance in electrophoretic analysis.



They reported a marked decrease in circulating albumin, a slight decrease in  $\gamma$ -globulin and essentially no change in  $\alpha$ -globulin. Because of the decreased plasma volume, the concentration of plasma  $\alpha$ -globulin was slightly increased in the depleted dogs. Studies were needed, therefore, to evaluate the effects of repletion on the several fractions of plasma proteins as revealed by electrophoretic analysis, so that correlations could be made between the total circulating protein fractions and the kind and amount of "protein" in the diet. Two enzymatic hydrolyzates, one of casein<sup>2</sup> with a nitrogen balance index in normal dogs of 0.8 and the other of lactalbumin (12) with a nitrogen balance index of 1.0, were fed to replete the dogs (10).

### METHODS

Dogs were made hypoproteinemic by feeding a protein-free diet and by plasmapheresis according to the method described by Seeley (9). This method in brief is as follows: Normal dogs were placed on a protein-free diet (13) consisting of 21.6% sucrose, 19.4% dextrose, 32.9% dextrin, 21.8% lard, 1.8% Wesson's modified Osborne-Mendel salt mixture, and 2.5% agar. The following vitamins were included: thiamine, riboflavin, nicotinic acid, calcium pantothenate, pyridoxine, choline 2-methyl-1-naphthoquinone and vitamins A and D. The dogs received 70 calories/day/kg. body weight. After one week of partial depletion of protein reserves by protein-free feeding, plasmapheresis was performed daily for four days.<sup>3</sup> During these days, one quarter of the blood volume was removed daily, the red cells being returned in an equivalent volume of physiological saline. Following this process of rapid depletion, the dogs were depleted further by feeding the protein-free diet until the plasma albumin-globulin ratio, determined electrophoretically, was reduced to approximately 0.22. The whole process of depletion required from 6 to 8 weeks for dogs which had been well fed and had abundant protein reserves. The dogs were repleted by feeding 6.6 g. of casein or lactalbumin hydrolyzate nitrogen/day/m.<sup>2</sup> of body surface for 30 days.

Determinations of plasma volume and plasma protein concentrations, as well as electrophoretic analyses of plasma, were performed before and after protein depletion, and also every 5 days during 30 days of repletion on protein hydrolyzate. It was thus possible to study the changes of each plasma protein component quantitatively during the course of protein hydrolyzate therapy. For electrophoretic analysis about 10 ml. of plasma was diluted with an equal volume of a barbiturate buffer of pH 8.4, and an ionic strength of 0.1 and dialyzed with 2 liters of the same buffer. The technique of Longworth (14) was used for the resolution of various components. Plasma volume was determined by the dye method of Gregerson and Stewart (15) and the plasma protein concentration was determined by micro Kjeldahl analysis.

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<sup>2</sup> Casein Hydrolysate, Squibb.

<sup>3</sup> The frequency of plasmapheresis depends upon the magnitude of the protein stores.

## RESULTS

### *Repletion with Casein Hydrolyzate*

Five dogs depleted in proteins were repleted with the casein hydrolyzate. Fig. 1 illustrates a typical series of electrophoretic patterns of

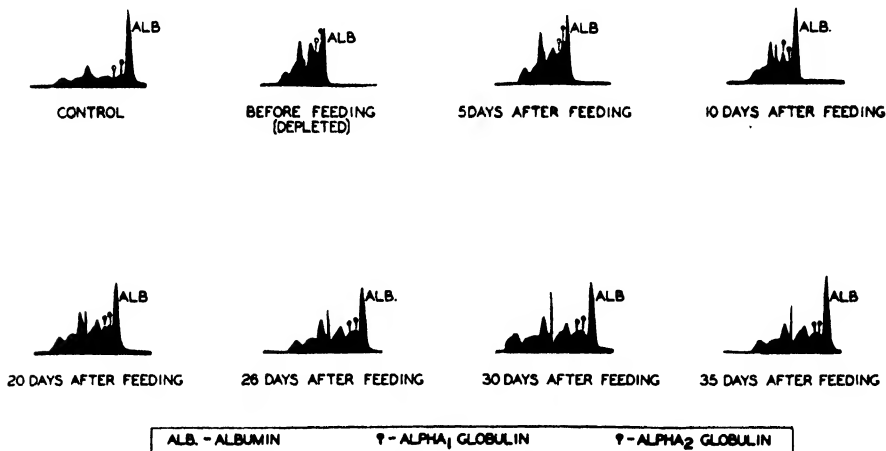


FIG. 1. The descending patterns of plasma of dog 68-1, showing the effect of protein depletion and repletion after oral administration of casein hydrolyzate.

plasma (Dog No. 68-1) taken before and after depletion, and during repletion with casein hydrolyzate. These patterns were recorded every 5 days during the repletion period. Each pattern is divided arbitrarily into 8 components. The patterns in Fig. 1 demonstrate that the albumin,  $\alpha$ -globulins, and  $\gamma$ -globulin separate into distinct peaks possessing relatively constant electrical mobilities. These components, therefore, can be identified easily and measured accurately. On the other hand, the remaining 4 components cannot be identified or measured so accurately, but are composed primarily of the  $\beta$ -globulins and fibrinogen.<sup>4</sup> The outstanding differences in pattern 1 of the normal plasma and pattern 2 of the plasma from the depleted dog are a decrease in albumin and an increase in  $\alpha$ -globulin fractions. These data agree with our previous findings that depletion of protein results in a decrease in the percentage of albumin and an increase in the percentage

<sup>4</sup> These four components will be referred to as "other globulins" throughout this paper.

of  $\alpha$ -globulins. Patterns 3, 4, 5, 6, show that the area under the albumin peak increases in proportion to the others and approaches the control value as feeding of the casein hydrolyzate was continued.

Since protein depletion generally results, however, in a decrease in plasma volume (16, 17) the changes in plasma proteins during depletion and repletion must be compared on the basis of total circulating proteins rather than on concentrations used for the patterns in Fig. 1. The data in Table I record the results on the restoration of total

TABLE I  
*The Effect of Oral Feeding of Casein Hydrolyzate on the Total Circulating Albumin and Globulins of Dogs Depleted in Proteins*

	Albumin Per cent $\pm$ stand. error	Globulin Per cent $\pm$ stand. error
Control	100	100
Depleted	29.2 $\pm$ 2.1	102 $\pm$ 4.7
1st Period	44.5 $\pm$ 5.6	107.3 $\pm$ 11.5
2nd Period	65.2 $\pm$ 4.4	121.5 $\pm$ 10.6
3rd Period	79.0 $\pm$ 7.5	113.0 $\pm$ 10.7
4th Period	82.6 $\pm$ 8.3	126.4 $\pm$ 9.6
5th Period	85.4 $\pm$ 9.8	131.0 $\pm$ 9.6
6th Period	95.4 $\pm$ 10.2	136.2 $\pm$ 9.1

circulating plasma albumin and globulins in 5 dogs orally fed with the casein hydrolyzate. The change of the total circulating protein is recorded percentagewise, with the amount present before depletion (called the control period) as 100. These data demonstrate that depletion in proteins causes a marked decrease in total circulating albumin in all dogs. On the other hand, the total circulating globulins are essentially unchanged.

The data also show that oral feeding of casein hydrolyzate caused a significant increase in total circulating albumin as well as globulin fractions in the plasma. In general, these increases are noticeable after the first or the second period of feeding. Although, after 6–7 periods of hydrolyzate feeding, *i.e.*, about 28–31 days, the average of the total circulating albumin of 5 dogs appears to approach 100, actually 3 dogs equaled or exceeded the control values, and the remaining 2 dogs reached only 75% of the control values. The amount of total circulating globulins increased and exceeded the control values in all dogs. Thus, repletion of dogs with casein hydrolyzate restores the major portion of albumin in most dogs and increases the globulins in all.

The effects of depletion and of repletion with casein hydrolyzate on the fractions of plasma globulin are shown in Table II. The total circulating  $\alpha$ - and  $\beta$ -globulins were not decreased by depletion. The  $\alpha$ - and the "other globulin" fractions can be reduced however by prolonged applications of plasmapheresis and protein-free feeding. Preliminary observations in our laboratories on these dogs indicate that they have suffered irreversible damage to the liver and to other organs. This may also be a factor in conditioning the response of human suffer-

TABLE II  
*The Effect of Oral Feeding of Casein Hydrolyzate on the Globulin Fractions of Dogs Depleted in Proteins*

	$\alpha$ -Globulins	Other globulin	$\gamma$ -Globulin
	<i>Per cent <math>\pm</math> stand. error</i>	<i>Per cent <math>\pm</math> stand. error</i>	<i>Per cent <math>\pm</math> stand. error</i>
Control	100	100	100
Depleted	118.6 $\pm$ 11.8	99.6 $\pm$ 9.7	78.4 $\pm$ 8.84
1st Period	128.0 $\pm$ 16.6	114 $\pm$ 15.0	69 $\pm$ 11.4
2nd Period	131 $\pm$ 12.5	126.2 $\pm$ 13.2	85.2 $\pm$ 12.4
3rd Period	128.7 $\pm$ 11.4	130.3 $\pm$ 14.2	106 $\pm$ 16.7
4th Period	109.4 $\pm$ 15.9	137 $\pm$ 17.0	113.6 $\pm$ 16.4
5th Period	126 $\pm$ 7.6	134.4 $\pm$ 11.4	134.8 $\pm$ 36.4
6th Period	133.6 $\pm$ 12.9	142.4 $\pm$ 12.2	118.6 $\pm$ 20.0

ing from long term depletion. However, in the studies reported here, where depletion and repletion have been studied as a reversible phenomenon, such dogs have not been used. The  $\gamma$ -globulins are significantly lower than control values in all but one of the depleted dogs. Repletion with casein hydrolyzate caused no sustained change in  $\alpha$ -globulins but the  $\gamma$ -globulins were returned to or increased above control values. The "other globulins" were increased also above control levels in all the dogs during repletion. These results demonstrate that the increase in globulins during the feeding of casein hydrolyzate is the result of the appearance of excess  $\gamma$ -globulin and "other globulins."

#### *Repletion with Lactalbumin Hydrolyzate*

Six dogs depleted in proteins were repleted with lactalbumin hydrolyzate. The typical series of electrophoretic patterns of the plasma of one dog (No. 44-11) repleted with this hydrolyzate is il-

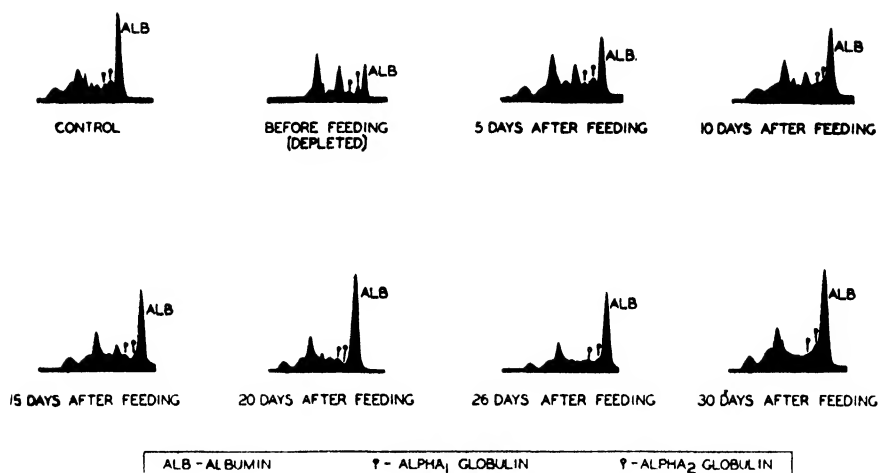


FIG. 2. The descending patterns of plasma of dog 44-11, showing the effect of protein depletion and repletion after the oral administration of lactalbumin hydrolyzate.

illustrated in Fig. 2. These patterns, like those in Fig. 1, demonstrate that depletion in proteins produces a decrease in per cent of albumin but an increase in the concentration of  $\alpha$ -globulins. Repletion with lactalbumin hydrolyzate causes an increase percentagewise in plasma albumin.

The quantitative results on the regeneration of the total circulating plasma albumin and globulins of these dogs fed orally with lactalbumin hydrolyzate are given in Table III. They also show a marked decrease in total circulating albumin, but only a slight decrease in total circulat-

TABLE III

*The Effect of Oral Feeding of Lactalbumin Hydrolyzate on the Total Circulating Albumin and Globulins of Dogs Depleted in Proteins*

	Albumin Per cent $\pm$ stand. error	Globulin Per cent $\pm$ stand. error
Control	100	100
Depleted	24.3 $\pm$ 2.3	93.8 $\pm$ 2.8
1st Period	39.3 $\pm$ 6.3	113 $\pm$ 5.6
2nd Period	55.8 $\pm$ 5.2	108.4 $\pm$ 5.9
3rd Period	72 $\pm$ 6.0	97.3 $\pm$ 6.9
4th Period	88 $\pm$ 7.7	91.7 $\pm$ 7.1
5th Period	88 $\pm$ 7.6	91.5 $\pm$ 5.3
6th Period	98.5 $\pm$ 7.3	101.7 $\pm$ 3.2
7th Period	104.4 $\pm$ 7.6	96 $\pm$ 4.8

ing globulin. Oral feeding of the lactalbumin hydrolyzate caused a gradual increase in total circulating albumin, reaching or exceeding control values in all dogs near the end of the repletion period. On the other hand, an initial rise in total circulating globulins is followed by a decrease so that, at the end of the repletion period, there is no significant increase of plasma globulin above control values. This initial rise in total circulating globulin can be compared with the observation of Whipple that feeding lactalbumin results in the formation of globulin when the dog is kept in the hypoproteinemic state by daily plasma-pheresis. Thus, the efficiency of dietary proteins on the restoration of

TABLE IV

*The Effect of Oral Feeding of Lactalbumin Hydrolyzate on the Globulin Fractions of Dogs Depleted in Proteins*

	$\alpha$ -Globulins	Other globulin	$\gamma$ -Globulin
	<i>Per cent <math>\pm</math> stand. error</i>	<i>Per cent <math>\pm</math> stand. error</i>	<i>Per cent <math>\pm</math> stand. error</i>
Control	100	100	100
Depleted	113.4 $\pm$ 9.5	92.3 $\pm$ 2.8	62.3 $\pm$ 7.2
1st Period	129 $\pm$ 10.5	119 $\pm$ 4.9	75.7 $\pm$ 10.3
2nd Period	135 $\pm$ 22.6	111.8 $\pm$ 4.8	75.6 $\pm$ 10.3
3rd Period	108.5 $\pm$ 14.6	102 $\pm$ 4.9	59.8 $\pm$ 7.3
4th Period	82.7 $\pm$ 15.7	101 $\pm$ 7.1	67 $\pm$ 5.4
5th Period	84.5 $\pm$ 7.3	92.5 $\pm$ 5.9	81.2 $\pm$ 6.5
6th Period	101.7 $\pm$ 5.2	104.8 $\pm$ 7.3	86.5 $\pm$ 10.0
7th Period	101.5 $\pm$ 6.4	97 $\pm$ 5.2	77.7 $\pm$ 10.4

plasma proteins is a function of the physiological state of the animal as well as of the type of protein fed. Prolonged repletion with lactalbumin hydrolyzate results in an increase in plasma albumin but not in total globulins in contrast to the casein hydrolyzate which restores both albumin and globulins.

The data in Table IV record the effects of depletion and of repletion with lactalbumin hydrolyzate on the various plasma globulins. The  $\gamma$ -globulin fraction is the only one which is decreased consistently in the protein depleted dog, and this increased somewhat with repletion by lactalbumin.

Repletion with the casein hydrolyzate was followed by the plasma albumin returning to control values. Similarly, the feeding of lactalbumin hydrolyzate was followed by the return of the plasma albumin

from an average of 6.9 g./m.<sup>2</sup> in the depleted condition to 29.5 g. in the repleted state, the latter value being 1.1 g. higher than the initial control value. Both groups of dogs showed no significant changes in the circulating  $\alpha$ -globulin on depletion or repletion. The group receiving casein hydrolyzate showed 9.1 g. of other globulin/m.<sup>2</sup> above the depleted levels, a 46% increase. The group of dogs receiving lactalbumin hydrolyzate did not show any significant increases in the

TABLE V

*The Average Total Circulating Albumin,  $\alpha$ -,  $\beta$ - and  $\gamma$ -Globulins in g./m.<sup>2</sup> of Body Surface and Albumin-Globulin Ratios in the Control, Depleted and Repleted Condition on 5 dogs repleted with a Casein Hydrolyzate and 6 Dogs Repleted with a Lactalbumin Hydrolyzate. Each dog received 0.55 g. of hydrolyzate nitrogen/kg. of body weight/day for 30 days.*

Condition	Circulating albumin	Circulating globulin			A/G
		Alpha	Gamma	Other	
	<i>g./m.<sup>2</sup></i>	<i>g./m.<sup>2</sup></i>	<i>g./m.<sup>2</sup></i>	<i>g./m.<sup>2</sup></i>	

Casein Hydrolyzate					
Control	25.7	9.7	4.8	19.6	0.75
Depleted	7.6	10.1	3.9	20.2	0.22
Repleted	23.4	10.9	6.0	28.7	0.51

Lactalbumin Hydrolyzate					
Control	28.4	8.1	6.0	21.5	0.80
Depleted	6.9	8.0	3.8	21.4	0.21
Repleted	29.5	7.3	5.3	22.3	0.85

"other globulins." Both groups of dogs, whether receiving the casein or the lactalbumin hydrolyzate, regenerated more  $\gamma$ -globulin during repletion than before, the casein hydrolyzate apparently being more effective in this respect.

The changes in albumin-globulin ratio (Table V) reflect the differences between the casein and lactalbumin hydrolyzate. The control ratio in the dogs which were repleted on casein hydrolyzate was 0.75, during depletion it decreased to 0.22, and after repletion the ratio only returned to 0.51 due to high globulin values. The control ratio of dogs

repleted on lactalbumin hydrolyzate was 0.80, during depletion it decreased to 0.21, and after repletion the ratio increased to 0.85.

It can be concluded that casein and lactalbumin hydrolyzates are equally effective in restoring plasma albumin and  $\gamma$ -globulins, but that casein is superior to lactalbumin in leading to an increase in "other globulins." Measured in terms of protein efficiencies<sup>5</sup> in growing rats or in nitrogen balance indexes (10) in normal dogs, lactalbumin hydrolyzate is superior to casein hydrolyzate. Thus, these two hydrolyzates are evaluated differently according to the biological process used to measure their effects upon the living system. These different evaluations give support to the concept that any one pattern of amino acids cannot be equally useful for all the purposes of protein metabolism in a complex organism.

### SUMMARY

Eleven dogs were depleted of protein by feeding a protein-free diet and by plasmapheresis. It was found that protein depletion resulted in marked decrease in the total circulating albumin and  $\gamma$ -globulins, whereas,  $\alpha$ - and "other globulins" remained unchanged.

The oral administration of casein hydrolyzate to five of these depleted dogs brought about an increase of both total circulating albumin and globulins; the latter was raised to even greater than control values. The other six dogs were fed a lactalbumin hydrolyzate. During the first ten days of feeding there was an increase in both albumin and globulin fractions. However, on prolonged feeding (about 31 days) the increase of total circulating albumin continued, whereas, the amount of total circulating globulins decreased. The difference in the plasma protein regeneration properties of these two hydrolyzates is discussed.

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# Desthiobiotin and O-Heterobiotin as Growth Factors for "Normal" and "Degenerate" Strains of *Clostridia*

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Received July 21, 1947

## INTRODUCTION

The role of biotin as a growth factor for the butyl alcohol producing anaerobes has been reported by Snell and Williams (1939) following the earlier work of Woolley, McDaniel and Peterson (1939), Weizmann and Rosenfeld (1939), and others. Lampen and Peterson (1941, 1943) showed that certain of these organisms required both *p*-aminobenzoic acid and biotin. They also studied (1943) the ability of a series of compounds related to *p*-aminobenzoic acid to replace it in the growth factor requirements of *Clostridium acetobutylicum*, but did not do the same for biotin. The biotin activity of desthiobiotin for yeast has been reported by Melville *et al.* (1943), and for other organisms by Leonian and Lilly (1944, 1945a). More recently Pilgrim *et al.* (1945) and Duschinsky *et al.* (1945) have reported that an oxygen analog of biotin has biotin-like activity for all facultative organisms tested which required biotin.

Extension of these studies to the anaerobes was tried in an effort to find organisms which could not utilize these substances as biotin substitutes, and to investigate the biotin requirements of "normal" and "degenerate" strains.

"Degeneration" in *Clostridia* has been known for some time as it was observed that certain anaerobes show altered characteristics (such as decreased solvents and riboflavin production) when transferred at frequent intervals for 10 or 20 transfers under conditions which do not allow sporulation to occur as usual. This has been reported by McCoy and Fred (1941), Williams (1936), Rodgers (1939), and others. Phelps (1941) made a comparative biochemical study of normal and "degenerate" cultures. He was unable to detect any fundamental differences in the culture to which the degenerative changes could be attributed.

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## EXPERIMENTAL

The cultures used in these experiments are in the collection of the Department of Agricultural Bacteriology, University of Wisconsin, and are numbered as in that collection. The bacteriological techniques described by Lampen and Peterson (1943) were followed. The stock cultures were kept on sterile soil and loop transfers were made to test tubes containing an inoculum medium consisting of the basal medium (listed below) to which 0.25% peptone and 0.25% yeast extract had been added. Two transfers were made on this medium at 24-hour intervals and the second transfer from the soil stock was centrifuged and the collected cells resuspended in an equal volume of freshly sterilized basal medium (ca. 10 ml.). Two drops of this cell suspension were added to each tube. All experiments were done in duplicate.

The basal medium contained (per liter): 20 g. glucose; 1 g.  $(\text{NH}_4)_2\text{HPO}_4$ ; 2 g. ammonium acetate; 1 g. salts (Lampen and Peterson (1943)); and 50 mg.  $\text{Na}_2\text{S}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$ . The medium for *Cl. felsineum* A-41 and *Cl. acetobutylicum* D also contained 50 mg./l. of *p*-aminobenzoic acid. The medium was adjusted to pH 6.8 and aliquots were placed in 18 × 150 mm. test tubes containing about 5 mg. reduced iron. The samples of biotin and other materials were added to the tubes and the final volume of medium in each tube was adjusted to 10 ml. The tubes were plugged with non-absorbent cotton and autoclaved at 127°C. for 15 minutes. Immediately upon cooling the tubes were inoculated and incubated at 30°C. in anaerobic jars. After 60 hrs. incubation, the growth was measured with an Evelyn photoelectric colorimeter (660 m $\mu$  filter) adjusted so that the uninoculated medium gave a reading of 100% transmission.

"Degeneration" of the cultures was induced by repeated transfers of the organisms at 24-hr. intervals on the inoculum medium. The inoculum from one transfer to the next in this series was approximately 3% of the volume. After 5 transfers under these conditions very few spores were observed, and after 10 transfers microscopic examination indicated the absence of spores. The organisms did not withstand heat shocking for one minute at 90°C. after the seventh transfer.

TABLE I  
Growth of "Normal" and "Degenerate" Cultures on  
Biotin-Free Basal Medium

Microorganism	First transfer	Tenth transfer	Twentieth transfer
	Per cent transmission	Per cent transmission	Per cent transmission
<i>Clostridium pasteurianum</i> A-65	84	82	70
<i>Clostridium pasteurianum</i> A-5	65	—	—
<i>Clostridium butylicum</i> A-21	100	79	66
<i>Clostridium butylicum</i> A-37	100	78	72
<i>Bacillus butylicus</i> A-39	89	75	75
<i>Clostridium felsineum</i> A-41	100	—	—
<i>Clostridium beijerinckii</i> A-68	85	60	—
<i>Bacillus saccharobutylicus</i> A-75	99	—	—
<i>Clostridium acetobutylicum</i> D	100	71	57

Although biotin is required for maximum growth by all of the cultures (normal and degenerate forms), an appreciable amount of growth is obtained in its absence by some of them. As indicated in Table I, the "degenerate" cultures seem to grow quite well on the biotin-free medium. This is not entirely in agreement with the similar experiment observed by Housewright and Koser (1944) who found that the *p*-aminobenzoic acid requirement of *Cl. acetobutylicum* did not change appreciably when the culture was transferred seven times without heat shock.

In Table II are summarized the results obtained when *d*-desthiobiotin

TABLE II  
Growth-Promoting Activity of Desthiobiotin and O-Heterobiotin

Microorganism	Biotin activity (Per cent)			
	<i>d</i> -desthiobiotin	<i>dl</i> -O-heterobiotin	<i>d</i> -biotin, <i>d</i> -desthiobiotin mixtures <sup>a</sup>	<i>d</i> -biotin, <i>dl</i> -O-heterobiotin mixtures <sup>b</sup>
<i>Clostridium pasteurianum</i> A-5	90	20	105	100
<i>Clostridium butylicum</i> A-21	100	50	100	120
<i>Clostridium butylicum</i> A-37	20	60	110	150
<i>Bacillus butylicus</i> A-39	10	5	100	100
<i>Clostridium felsineum</i> A-41	30	0	100	100
<i>Clostridium pasteurianum</i> A-65	30	15	50	65
<i>Clostridium beijerinckii</i> A-68	90	15	60	60
<i>Bacillus saccharobutylicus</i> A-75	100	60	105	140
<i>Clostridium acetobutylicum</i> D	120	30	100	110

<sup>a</sup> Mixtures contained 50% *d*-biotin and 50% *d*-desthiobiotin.

<sup>b</sup> Mixtures contained 33% *d*-biotin and 67% *dl*-O-heterobiotin.

tin, *dl*-O-heterobiotin, mixtures of *d*-biotin and *d*-desthiobiotin, and mixtures of *d*-biotin and *dl*-O-heterobiotin were added to the basal medium inoculated with the "normal" cultures. The growth response obtained when increasing amounts of *d*-biotin and substitutes were added to the medium was used to form a curve (range from 10 mγ to 500 mγ/l.), and the average *d*-biotin equivalent for the other compounds and mixtures was calculated from the biotin curve and is indicated in the table. The *dl* form of O-heterobiotin was used in these experiments, but only the *d* form was assumed to have activity. Data obtained in similar experiments with the "degenerate" culture are found in Table III.

Several of the "normal" cultures were transferred at 3 day intervals with heat shocking for 3 minutes at 90°C. to kill vegetative cells. After some 10 transfers the biotin requirements were nearly identical with that of the first transfer from the spore stocks.

The "anti" biotin and "anti" desthiobiotin effect of O-heterobiotin for "normal" cultures of *Cl. felsineum* A-41 and *Bacillus butylicus* A-39 was tested. While the results were somewhat unexpected, in that at certain combinations of desthiobiotin and O-heterobiotin growth was

TABLE III  
Effect of Continued Transfer of Cultures on Growth-Promoting  
Activity of Desthiobiotin and O-heterobiotin

Microorganism	Biotin activity (Per cent)					
	<i>d</i> -desthiobiotin		<i>dl</i> -O-heterobiotin		<i>d</i> -biotin and <i>dl</i> -O-heterobiotin mixtures <sup>c</sup>	
	A <sup>a</sup>	B <sup>b</sup>	A	B	A	B
<i>Clostridium butylicum</i> A-21	100	30	40	40	40	40
<i>Clostridium butylicum</i> A-37	20	0	35	20	30	35
<i>Bacillus butylicus</i> A-39	15	100	45	40	100	100
<i>Clostridium pasteurianum</i> A-65	75	85	40	20	100	100
<i>Clostridium beijerinckii</i> A-68	100	—	50	—	100	—
<i>Clostridium acetobutylicum</i> D	95	75	55	45	100	80

<sup>a</sup> Tenth transfer from spores.

<sup>b</sup> Twentieth transfer from spores.

<sup>c</sup> Mixture contained 33% *d*-biotin and 67% *dl*-O-heterobiotin.

obtained while at higher and lower concentrations it was not, the general result seemed to show that O-heterobiotin will antagonize biotin at a ratio of 20,000, and desthiobiotin at 200,000. This does not appear to be a toxic effect as increasing the *d*-biotin concentration immediately overcomes this inhibition.

#### OTHER CHARACTERISTICS OF "NORMAL" AND "DEGENERATE" CULTURES

Concurrent with the observations on the biotin requirements of the cultures, the solvents and riboflavin production were tested in a semi-quantitative way. "Degeneration" in the twelfth transfer was indi-

cated by a reduction in the solvents production and the pH of the fermentation remained about pH 5. The data of Rodgers (1939) and Phelps (1941) indicate that considerable variation often occurs in acid production by degenerate cultures and this is not to be regarded as a quantitative indication of the extent of degeneration. Rodgers (1939) also noticed that riboflavin synthesis decreased rapidly as the culture degenerated. The riboflavin synthesized by the "degenerate" cultures studied in these experiments (twentieth transfers) was a fraction of 1% of that produced by the parent "normal" cultures.

It was noticed that the streptomycin sensitivity of these "degenerate" cultures was also different from that of the "normal" parent. The minimum concentration of streptomycin inhibiting growth, which was approximately 2  $\gamma$ /ml. at the first transfer, increased to approximately 1 mg./ml. on the twentieth transfer. Penicillin sensitivity of the "degenerate" cultures was not different from that of the parent culture; 0.2 u. of penicillin G/ml. of medium was the minimal inhibitory concentration for "normal" and "degenerate" cultures. Strain and species variation was not observed among the organisms tested. These experiments indicate that streptomycin sensitivity is another qualification for differentiating "degenerate" and "normal" strains.

### DISCUSSION

As indicated in Table II, some of the organisms, including *Cl. butylicum* A-21 and *B. saccharobutylicus* A-75, are able to utilize either desthiobiotin or O-heterobiotin nearly as well as biotin. Desthiobiotin seems to have the same activity as biotin for *Cl. pasteurianum* A-5, *Cl. acetobutylicum* D, and *Cl. beijerinckii* A-68, but O-heterobiotin does not quite have equal potency with biotin for these organisms. *Cl. felsineum* A-41 and *B. butylicus* A-39 do not seem to utilize O-heterobiotin as a replacement for biotin, whereas desthiobiotin has slight activity for these organisms. Both desthiobiotin and O-heterobiotin have slight activity for *Cl. pasteurianum* A-65, while *Cl. butylicum* A-37 seems to utilize the O-heterobiotin equally as well as biotin, but desthiobiotin has only slight activity for this organism. Thus, it seems possible to find anaerobes which under normal conditions of growth are able to utilize either desthiobiotin or O-heterobiotin, or neither of these compounds, as substitutes or partial substitutes for biotin. Winnick *et al.* have noted that oxybiotin (O-heterobiotin) is not

utilized as completely as biotin by *Rhizobium trifolii* and other organisms.

The utilization of mixtures of O-heterobiotin and biotin and mixtures of desthiobiotin and biotin by some organisms not utilizing O-heterobiotin or desthiobiotin (respectively) to a very high degree is a curious circumstance. It has also been observed in experiments with *Memnoniella echinata* (unpublished). The activity indicated in the tables is based on the activity of the mixtures as compared with the activity of an equal quantity of biotin. Conversion of these analogs to biotin necessitates isolation for effective proof; microbiological assays of treated materials vary considerably with the assay organism, as well illustrated by Leonian and Lilly (1945b). A similar phenomenon with mixtures of riboflavin and other flavins was reported by Snell and Strong (1939).

#### ACKNOWLEDGMENTS

I am indebted to Drs. S. A. Harris and W. R. Sullivan for the biotin analogs, to Drs. E. McCoy and H. J. Koepsell for certain of the cultures, and to Drs. A. E. Axelrod, J. O. Lampen, and W. H. Peterson for constructive comments. Certain laboratory facilities were made available through Dr. J. W. Foster.

#### CONCLUSIONS

Desthiobiotin and O-heterobiotin may replace biotin as a growth factor for certain *Clostridia*. Mixtures of biotin and desthiobiotin, and mixtures of biotin and O-heterobiotin are, in general, as active as the corresponding amount of biotin.

"Degenerate" cultures produced by rapid serial transfer of these *Clostridia* do not have the same biotin requirements as the normal cultures, and appreciable growth is obtained in the absence of biotin. Utilization of related compounds as substitutes for biotin by the "degenerate" cultures varies from utilization by the "normal" cultures.

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# The Hexosemonophosphatase System (Glucose-6-Phosphatase) of Liver<sup>1</sup>

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With the technical assistance of

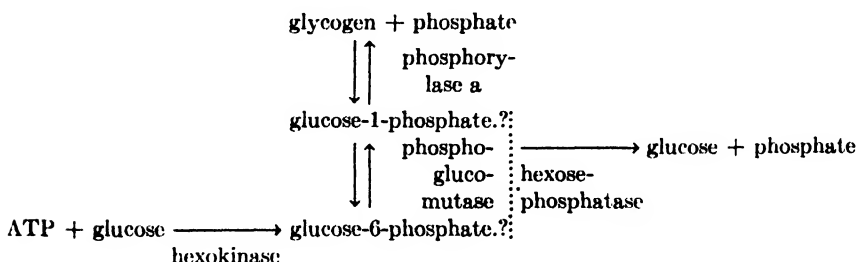
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Received July 28, 1947

## INTRODUCTION

The balance between the rates of hepatic glycogenesis and glycogenolysis determines the rate of glucose secretion to the blood. The mechanisms involved in these cycles have been clarified largely as the result of the investigations of Cori and his collaborators. In consequence of their studies, the glycogenic and glycogenolytic cycles of the liver can be depicted as follows:



However, the mechanism of the transformation of hexosemonophosphate to blood glucose is still obscure. Cori, Cori and Schmidt (1) demonstrated that when glucose-1-phosphate was added to liver extract, part of it was accounted for as a result of the formation of free glucose, whereas the remainder was converted to glucose-6-phosphate. Consequently, they were unable to decide whether glucose-1-phos-

<sup>1</sup> Aided in part by a grant from the Eli Lilly Company.

phate was itself directly dephosphorylated or whether it was transformed first to glucose-6-phosphate prior to dephosphorylation. Ostern *et al.* (2) also investigated this aspect of the glycogenolytic cycle and concluded that, in the natural course of the reaction, glucose-1-phosphate probably underwent direct dephosphorylation. We have investigated the activity of the hexosemonophosphatase system of the liver and obtained data which indicate that it is glucose-6-phosphate rather than glucose-1-phosphate which is subjected to dephosphorylation during the glycogenolytic cycle in the liver.

### METHODS

The liver extracts used in this investigation were obtained from either well fed rats or rabbits. During the early part of this work, the liver extracts were also used as the source of enzymes other than those concerned with the transformation of glucose-1-phosphate to glucose and inorganic phosphate and fresh extracts were prepared from rats in the following manner: The animals were stunned by a blow on the head, bled from the neck vessels and the liver rapidly removed, freed from the gall bladder, weighed in an ice cold dish, minced with scissors and ground for 5 minutes in a mortar and pestle with 1.4 volumes of ice cold water. The mixture was then centrifuged for 5 minutes at high speed and the supernatant fluid filtered through gauze. The extract thus obtained was used immediately after it had been allowed to come to the temperature used for incubation.

Later in the work, when need no longer existed for the use of such fresh extracts, and after it had become apparent that the systems involved in the conversion of glucose-1-phosphate to glucose were quite stable over an extended period of storage, a large quantity of extract was prepared from the liver of a well fed rabbit which was sacrificed by the intravenous injection of air and bled rapidly from the severed neck vessels. The liver was removed rapidly, weighed and blended immediately for 5 minutes with two volumes of ice cold water in a Waring Blender. The resulting mixture was centrifuged at high speed for 5 minutes and the supernatant filtered through gauze. The extract thus obtained was divided into 10 ml. lots and stored at  $-45^{\circ}\text{C}$ . The activity of the system concerned in the dephosphorylation of hexosemonophosphate proved to be quite stable in such preparations over a period of at least two months. Each individual lot of frozen extract was allowed to thaw just prior to its usage.

The glucose-1-phosphate used in this work was a preparation of the dipotassium salt more than 95% pure. The activity of the hexosemonophosphatase system was assayed by estimation of either or both of the end products of this reaction. Inorganic phosphate was determined by a modification of the method of Fiske and Subbarow (3) adapted to the use of the photoelectric colorimeter. The easily hydrolyzable phosphate was determined by analysis for the inorganic phosphate after seven minutes of hydrolysis at  $100^{\circ}\text{C}$ . with 1 *N* hydrochloric acid ( $P_7$ ) and subtraction from this value of the inorganic phosphate content prior to hydrolysis ( $P_7-P_0$ ). Glucose was determined by the Nelson (4) procedure.

For most purposes, the amount of hexose-6-phosphate present was calculated from the difference of the  $P_7$  values ( $-\Delta P_7$ ) before and after incubation. In a few cases, the validity for this calculation was verified by simultaneous determination of the total phosphate in order to calculate the amount of difficultly hydrolyzable phosphate. However, inasmuch as the transformation of glucose-1-phosphate to hexose-6-phosphate involves the conversion of an easily hydrolyzable phosphate group to one which is not hydrolyzed during 7 minutes at  $100^\circ\text{C}$ . in 1 *N* HCl, this transformation is reflected by the decrease in the  $P_7$  values of the reaction mixture. Due to the presence in the liver extract of phosphate esters other than the one added during the experiment, control tests with the extracts incubated alone without added hexosemonophosphate always showed some small increase in both the  $P_0$  and  $P_7$  values. However, in the presence of added hexosemonophosphate, these changes were so low in comparison with those produced as the result of the breakdown of the hexosemonophosphate that they could be, for all practical purposes, neglected and the calculations described above could be used with justification to follow the course of the reaction.

The various constituents to be added to the reaction mixture were mixed and allowed to come to the temperature of incubation. After the liver extract had also come to equilibrium at the desired temperature, the appropriate quantity was added. The moment of its addition was taken as the zero time for incubation. Analyses for the composition of the reaction mixtures at zero time were carried out by adding comparable proportions of the constituents directly to trichloroacetic acid, for the determination of phosphate, and to barium hydroxide for the determination of glucose. During the course of incubation, samples were withdrawn at the specified times and analyzed as described above. Incubation was always performed at  $30^\circ\text{C}$ . Aerobic conditions were obtained by the use of test tubes exposed to the atmosphere. Anaerobic conditions were maintained by incubation in Warburg flasks in an atmosphere of 95%  $\text{N}_2$ -5%  $\text{CO}_2$  with a bicarbonate buffer at pH 7.5.

The other buffers used for aerobic incubation were those commonly described. In most of the experiments cited, veronal buffer at pH 7.5, as described by Michaelis (5), was utilized. Whenever various substances were added to the reaction mixture, a description of such is found in the appropriate protocol.

## RESULTS

Previous demonstrations (1, 2) of the ability of liver extracts to promote the conversion of glucose-1-phosphate to glucose and inorganic phosphate were readily confirmed. In contrast to the experience of Colowick, Welch and Cori (6), who noted that "aerobically, the dephosphorylation of the added monoester was masked by a rapid reesterification of the liberated inorganic P," with the extracts and conditions used in the present investigation, the courses of the reactions were similar under both aerobic and anaerobic conditions (Table I).

Erroneous conclusions concerning the mechanism of this conversion may be drawn unless repeated examinations are made during the

TABLE I

*Action of Liver Extracts on Glucose-1-Phosphate Under Aerobic and Anaerobic Conditions*

Aerobic incubation with veronal buffer, pH 7.5. Anaerobic incubation in 5% CO<sub>2</sub>-95% N<sub>2</sub> in bicarbonate buffer, pH 7.5. Rat liver extracts. Initial concentration of glucose-1-phosphate = 0.008 M. Incubation time 30 minutes.

Gas phase	Phosphate changes during incubation					
	P <sub>0</sub> ( $\gamma$ /ml.)			P <sub>i</sub> ( $\gamma$ /ml.)		Glucose-6-phosphate at end of incubation (expressed as $\gamma$ /ml. of difficultly hydrolyz- able phosphate)
	Before incubation	After incubation	Amount formed	Before incubation	After incubation	
Aerobic	30	193	163	260	206	54
Anaerobic	23	174	151	263	183	80
Aerobic	55	142	87	286	164	122
Anaerobic	22	132	110	260	145	115

course of the reaction. A single analysis at the end of a 30 minute incubation period reveals the almost complete disappearance of the added glucose-1-phosphate. Part of this has been converted to glucose-6-phosphate, whereas the remainder is accounted for by glucose and inorganic phosphate. Such findings suggested to Ostern *et al.* (2) the activity of two distinct mechanisms, one of which dephosphorylates the Cori ester and the other of which transforms the latter into hexose-6-phosphate. However, when samples for analysis are withdrawn from the reaction mixture at intervals, a clearer insight of the mechanism involved is obtained.

The results of such analyses are demonstrated graphically in Fig. 1, which illustrates the progressive formation of inorganic phosphate ion during the course of the reaction, and indicates that the reaction can be separated into two distinct phases. The first phase of the reaction is of short duration and involves a rapid decrease in the P<sub>i</sub> values for the reaction mixture. This indicates the conversion of glucose-1-phosphate to glucose-6-phosphate, a fact which has been verified by actual analysis for the presence of hexose-6-phosphate. The second phase of the reaction is characterized by an increase in the P<sub>i</sub> values and an almost exactly parallel increase in the P<sub>0</sub> values. These latter figures correspond to the conversion of the difficultly hydrolyzable phosphate

ester (hexose-6-phosphate) into free phosphate and glucose. It would appear that the major portion of the glucose-1-phosphate is transformed, during the early course of the reaction, first into glucose-6-phosphate, following which the latter undergoes dephosphorylation.

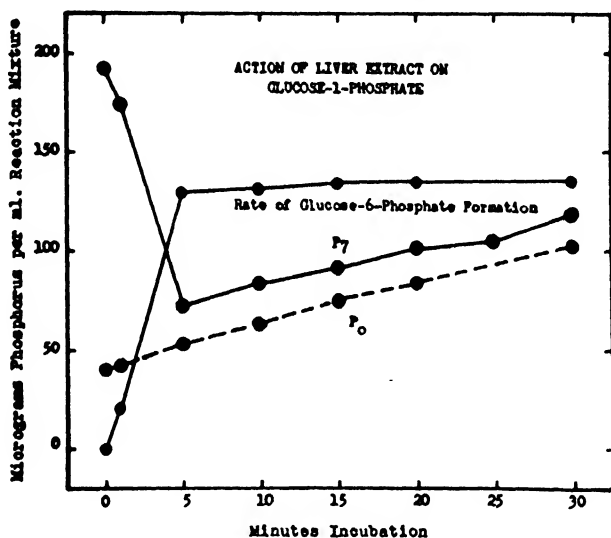


FIG. 1. Rabbit liver extract, aerobic incubation pH 7.5; glucose-1-phosphate 0.0049 *M*.

Based on figures obtained from analysis of their reaction mixture at the end of a 30-minute period of incubation which revealed a greater increase in inorganic phosphate than in the amount of hexose-6-phosphate present, Ostern *et al.* (2) concluded that the rate of formation of inorganic phosphate from Cori ester surpassed the rate of conversion of glucose-1-phosphate to glucose-6-phosphate. Our data reveal that such is not the case. On the other hand, the rapidity of the conversion of glucose-1-phosphate into the 6-ester agrees well with the observations of Colowick and Sutherland (7) concerning the kinetics of the phosphoglucomutase reaction since these latter investigators found that almost 95% of the Cori ester had been transformed into hexose-6-phosphate at the end of 10 minutes of incubation. Indeed, if the tacit assumption is made that all of the inorganic phosphate appearing in the reaction mixture is derived as a result of the dephosphorylation of hexose-6-phosphate and that none is formed from the direct hydrolysis of

glucose-1-phosphate (in other words, that the total amount of glucose-6-phosphate that has been formed is equal to the amount of the latter actually present plus that represented by the increase in inorganic phosphate), the data presented in Fig. 1 correspond well to those obtained by Colowick and Sutherland in their study of the phosphoglucomutase reaction. The curve drawn from such an assumption is illustrated in Fig. 1.

The close similarity between this curve and that for the phosphoglucomutase mechanism, together with the other facts revealed in Fig. 1, presents presumptive evidence that glucose-1-phosphate undergoes preliminary transformation into the 6-ester prior to the dephosphorylation of hexosemonophosphate. However, such results do not constitute indisputable evidence that Cori ester itself also is not dephosphorylated directly. Theoretically, at least two mechanisms may exist for the conversion of hexosemonophosphate to glucose. According to one mechanism, part of the Cori ester would be transformed into Robison ester and part dephosphorylated directly. Part of the hexose-6-phosphate so formed would also undergo dephosphorylation. Such a scheme would postulate the existence either of two specific hexosemonophosphatases, each of which acts only on its particular substrate or, alternatively, of a single hexosemonophosphatase capable of catalyzing the hydrolysis of both the 1- and 6-esters. The second scheme would postulate that the conversion of glucose-1-phosphate to hexose-6-phosphate must precede dephosphorylation and implies the existence of a specific hexose-6-phosphatase capable of promoting the hydrolysis only of the 6-ester.

The data obtained solely from a study of the course of the reaction cannot be used to establish a definite conclusion concerning the exact mode of the mechanism involved although they certainly do indicate that added glucose-1-phosphate is largely converted to glucose-6-phosphate prior to the formation of the greater part of the liberated inorganic phosphate. Nevertheless, since the phosphoglucomutase reaction has been demonstrated to be reversible (7), the possibility cannot be denied that, after the formation of hexose-6-phosphate, the latter is reconverted to the 1-ester, which in turn undergoes its own dephosphorylation. Furthermore, although a study of the course of the reaction definitely indicates that the greater part of the free phosphate is formed directly or indirectly from glucose-6-phosphate, the data do not preclude the possibility that the inorganic phosphate

appearing during the first phase of the reaction is derived from the direct dephosphorylation of glucose-1-phosphate. Accordingly, the effects of various agents which inhibit the breakdown of glucose-1-phosphate by liver extract were studied in an effort to obtain more insight into the mechanism involved in this transformation.

#### A. FLUORIDE ION

Sodium fluoride is known to inhibit the transformation of glucose-1-phosphate into glucose and inorganic phosphate. Ostern *et al.* (2) demonstrated that, in the presence of fluoride, the amount of inorganic phosphate formed from Cori ester was decreased and that this was accompanied by an increase in the amount of Robison ester present at the end of the period of incubation. They therefore concluded that fluoride inhibited the phosphatase acting directly on glucose-1-phosphate and thus permitted the formation of larger amounts of the

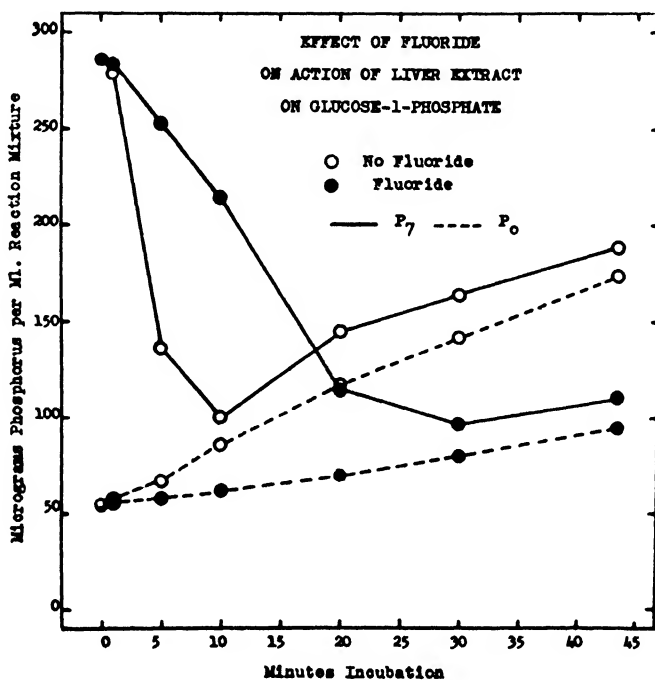


FIG. 2. Rat liver extract, aerobic incubation pH 7.5; glucose-1-phosphate 0.008 M; NaF 0.056 M.



6-ester than were formed in the absence of fluoride. However, an entirely different conclusion is reached when the course of the reaction is studied. Fig. 2 illustrates the kinetics of the reaction in the presence and absence of fluoride and reveals that the latter actually tends to inhibit the transformation of the 1-ester to the 6-ester. At the same time, it also inhibits the liberation of free phosphate from the hexose-6-phosphate so formed. In other words, instead of favoring the formation of larger amounts of Robison ester, as was concluded by Ostern *et al.*, fluoride actually tends to depress its formation. It is the marked inhibition of the breakdown of hexose-6-phosphate by fluoride that is responsible for the accumulation of the former in larger amounts in the reaction mixture.

The above can be demonstrated in another manner. When fluoride is added to the reaction mixture, after the conversion of the greater part of the glucose-1-phosphate to Robison ester had already occurred,

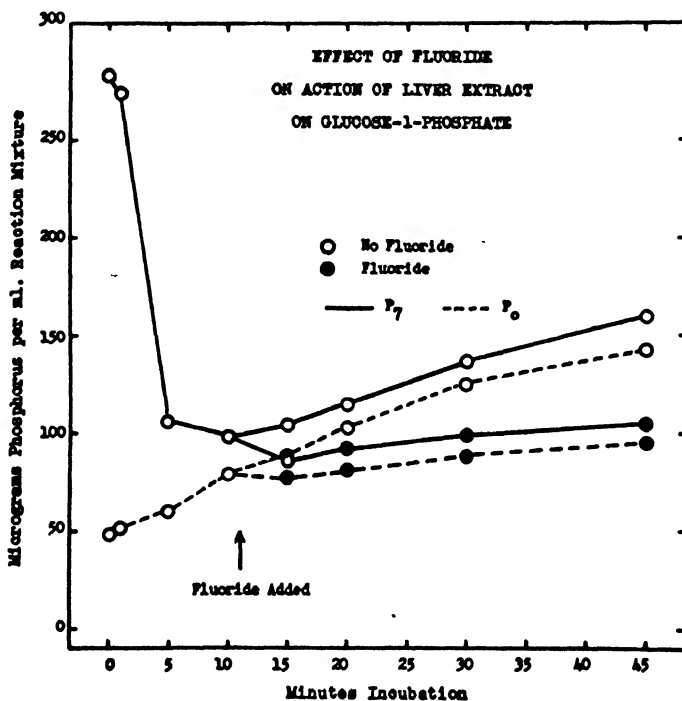


FIG. 3. Rat liver extract, aerobic incubation pH 7.5; glucose-1-phosphate 0.008 M; NaF added to a concentration of 0.056 M.

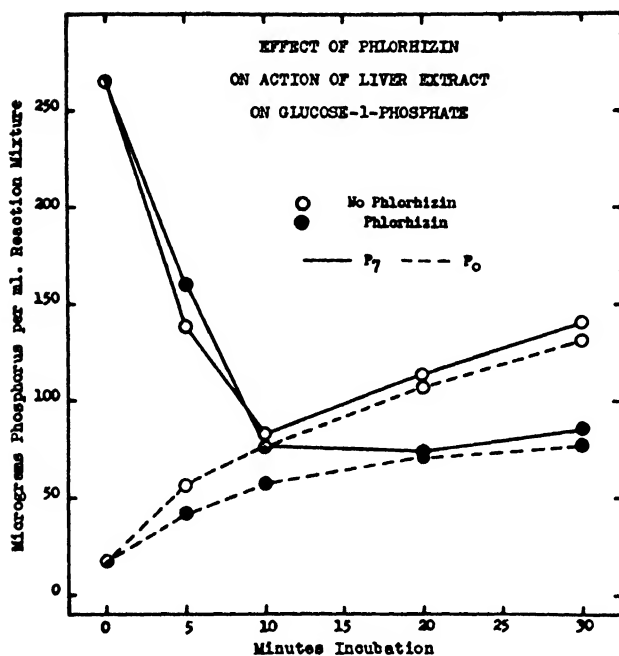


FIG. 4. Rat liver extract, anaerobic incubation pH 7.5 (bicarbonate buffer); glucose-1-phosphate 0.008 *M*; phlorizin 0.0126 *M*.

the liberation of inorganic phosphate from the latter is inhibited (Fig. 3).

### B. PHLORIZIN

Phlorizin is also known to be effective in the suppression of the formation of inorganic phosphate from added glucose-1-phosphate (1, 2). As with fluoride, the addition of phlorizin results in the accumulation of larger amounts of Robison ester in the reaction mixture. The data depicted in Fig. 4 indicate that this effect is due to the fact that, whereas the presence of phlorizin did not appreciably effect the transformation of the 1-ester to the 6-ester, the conversion of hexose-6-phosphate to inorganic phosphate was markedly inhibited.

### C. HEXOSES

Adequate concentrations of glucose have been demonstrated by Ostern *et al.* (2) to inhibit the breakdown of glucose-1-phosphate into

hexose and inorganic phosphate. The mechanism of this inhibition is demonstrated in Fig. 5 which reveals that glucose fails to influence the transformation of the 1- to the 6-ester but merely prevents the dephosphorylation of the latter. Thus, in its overall effect, the action of glucose resembles that of phlorizin. The effects of glucose are proportional to its concentration in the reaction mixture, increasing concentrations

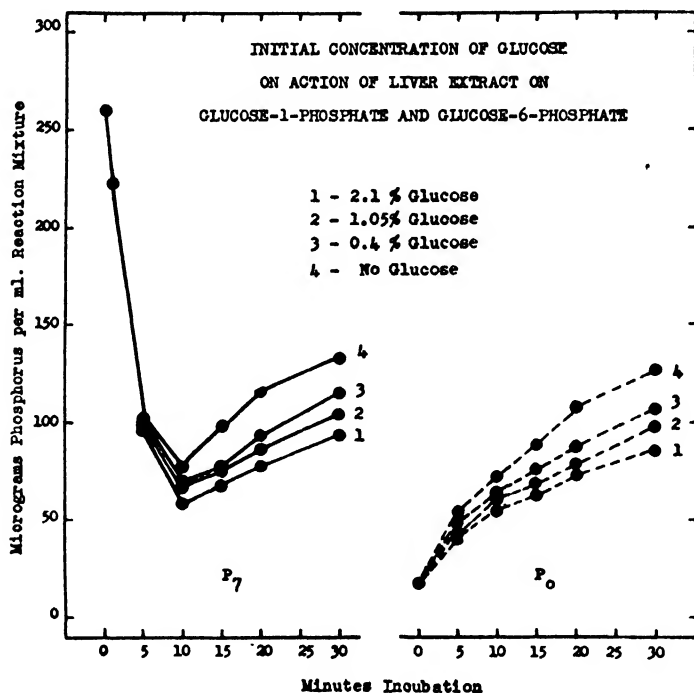


FIG. 5. Rat liver extract, aerobic incubation pH 7.5;  
glucose-1-phosphate 0.008 *M*.

producing successive increments in the amount of inhibition of the liberation of inorganic phosphate whereas all concentrations fail to affect the conversion of glucose-1-phosphate to the 6-ester.

Glucose proved to be the most effective of the hexoses investigated in producing this inhibition. Equal concentrations of glucose were much more inhibitory than galactose, mannose or fructose (Table II). Since glucose is one of the reaction products of the dephosphorylation of hexosephosphate, the possibility arises that its inhibitory activity

TABLE II

*The Action of Liver Extract on the Formation of Inorganic Phosphate from Glucose-1-Phosphate in the Presence of Various Hexoses*

Aerobic incubation, pH 7.5. The total initial volume of each reaction mixture was 7.2 ml. and contained 1.5 ml. of rabbit liver extract, 1.2 ml. of veronal buffer, 13 mg. of glucose-1-phosphate, and the hexoses in a final concentration of 2.1%.

Added hexose	Inorganic phosphate content of reaction mixture in $\gamma$ /ml.						
	Duration of incubation in minutes						
	0	1	5	10	15	20	30
None	23	31	42	56	65	72	89
Glucose	23	28	39	46	49	53	69
Fructose	23	28	43	54	62	72	87
Mannose	23	29	41	49	57	68	81
Galactose	23	31	43	54	64	70	91

might be attributed to a simple mass action effect; *i.e.*, an increase in the concentration of glucose tends to cause the dephosphorylative reaction to proceed in the reverse direction. However, all attempts to demonstrate the reversibility of this reaction have failed. Thus, liver extracts, in the presence of high concentrations of glucose and inorganic phosphate, appear unable to promote the phosphorylation of glucose since no accumulation of phosphate esters in the reaction mixture is noted even in the presence of insulin (Table III). Whereas the pos-

TABLE III

*The Irreversibility of the Hexosephosphatase Reaction*

Aerobic incubation, pH 7.5. The total initial volume of the reaction mixture was 7.2 ml. and contained 1.5 ml. of rabbit liver extract, 1.2 ml. of veronal buffer, glucose in a final concentration of 4.2% and, inorganic phosphate adjusted to pH 7.5 in a final concentration of 0.007 *M*. Incubation for 30 minutes.

Phosphate changes during incubation

Insulin	$P_0$ ( $\gamma$ /ml.)			$P_T$ ( $\gamma$ /ml.)		
	Before incubation	After incubation	Amount utilized	Before incubation	After incubation	Amount esterified
None	240	240	0	258	246	—12
1.2 mg.	240	244	—4	258	254	—4

sibility exists that such esters are formed but are immediately dephosphorylated, for all practical purposes the hexosemonophosphatase reaction appears to be unidirectional and proceeds only in the direction of the dephosphorylation of hexose phosphate.

Although inorganic phosphate is known to inhibit the dephosphorylation of glycerophosphates (8) and has been assumed (2) to inhibit the conversion of hexosemonophosphate to free glucose, such an effect has been difficult to demonstrate. Marked increases in the initial inorganic phosphate content of the reaction mixture do not appear to depress the formation of glucose during incubation as judged by comparison of the amounts of glucose appearing during incubation in phosphate and veronal buffers at pH 7.5 (Table IV). It is, of course,

TABLE IV

*Formation of Glucose from Glucose-1-Phosphate by Liver Extracts in Phosphate and Veronal Buffers*

Aerobic incubation, pH 7.5. The total initial volume of each reaction mixture was 3.6 ml. and contained 0.7 ml. of rabbit liver extract, 6.5 mg. of glucose-1-phosphate, and 0.6 ml. of veronal or Sørensen's phosphate buffer. Glucose content expressed as mg.-%. Time of incubation: 45 minutes.

Buffer	Glucose changes in reaction mixture		
	Before incubation	After incubation	Glucose formed
Veronal Phosphate	121	199	88
	121	205	94
Veronal Phosphate	93	160	67
	93	168	75

possible that still higher concentrations of phosphate might prove to be more inhibitory. Furthermore, the increase in the phosphate content might be expected to increase the rate of glucose-1-phosphate formation by phosphorylation of the glycogen present in the liver extracts. As a result, the increase in the concentration of glucose-1-phosphate would tend to increase the rate of formation of inorganic phosphate since the rate of formation of this latter from hexosephosphate increases with the initial concentration of the latter (Fig. 6). Hence, any inhibition due to phosphate might have been concealed as a result of the increased rate of dephosphorylation dependent on this latter factor. This question however, has not been further investigated.

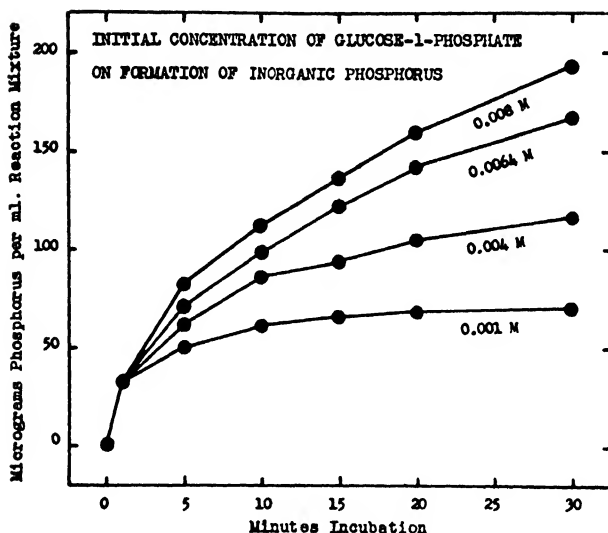


FIG. 6. Rat liver extract, aerobic incubation pH 7.5.

#### D. ZINC ION AND ALLOXAN

Lehmann (9) demonstrated that both zinc ion and alloxan inhibited the transformation of glucose-1-phosphate to the 6-ester by the phosphoglucomutase system of muscle extracts. The results in Fig. 7 demonstrate the same inhibiting action on the phosphoglucomutase system in liver extracts. Both 0.001 M  $Zn^{++}$  and 0.2% alloxan almost completely suppressed the transformation of the 1- into the 6-ester and at the same time prevented the liberation of inorganic phosphate.

The inhibitory action of zinc and alloxan may be due to one of several mechanisms. If the inorganic phosphate liberated is derived directly from the dephosphorylation of glucose-1-phosphate, they must inhibit the hexosephosphatase system acting on this latter compound. If the inorganic phosphate, on the other hand, is liberated solely as a result of the direct dephosphorylation of the 6-ester, their inhibiting action might be attributed either to their prevention of the formation of Robison ester from the glucose-1-phosphate or to their inhibition of the phosphatase acting on glucose-6-phosphate. Some insight into the actual mechanism was gained from a comparison of their effects on the transformations of added glucose-1-phosphate as contrasted to their effects when Robison ester was initially present in

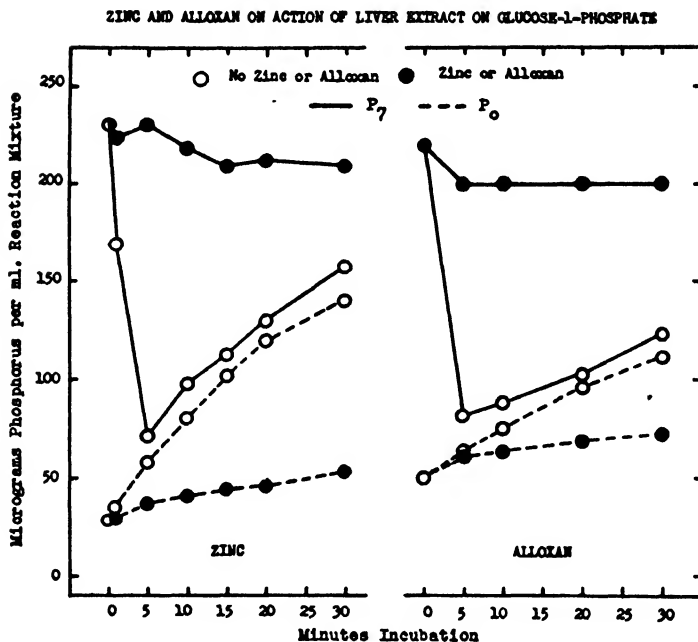


Fig. 7. Rat liver extract, aerobic incubation pH 7.5; glucose-1-phosphate 0.0064 *M*; ZnSO<sub>4</sub> 0.001 *M*.

Rabbit liver extract, aerobic incubation pH 7.5; glucose-1-phosphate 0.005 *M*; alloxan 0.2%.

the reaction mixture. The results of such a comparison are found in Table V, where it is seen that 0.001 *M* zinc ion and 0.2% alloxan cause 79% and 74%, respectively, inhibition of the formation of inorganic phosphate from glucose-1-phosphate. On the other hand, the same amounts of zinc and alloxan produced only 49% and 30%, respectively, inhibition of the formation of inorganic phosphate from Robinson ester. Such figures indicate that both of these agents inhibit not only the phosphoglucomutase reaction but also, to some extent, the dephosphorylation of glucose-6-phosphate. However, especially in the case of alloxan, the extent of the inhibition of the phosphoglucomutase reaction is so much greater and is so effective that it permits the formation of only very small quantities of glucose-6-phosphate. On the other hand, once glucose-6-phosphate has been formed, the rate of its dephosphorylation becomes appreciable, even in the presence of the inhibitors.

TABLE V

*The Effects of Zinc and Alloxan on the Action of Liver Extracts on Glucose-1-Phosphate and Glucose-6-Phosphate*

Aerobic incubation, pH 7.5. Initial concentration of glucose-1-phosphate 0.00485 M and glucose-6-phosphate 0.0025 M. Rabbit liver extract used with alloxan and rat liver extract with zinc sulfate. Incubation period 20 minutes.

Inhibitor	Substrate			
	Glucose-1-phosphate		Glucose-6-phosphate	
	Inorganic phosphate formed (γ/ml.)	Per cent inhibition	Inorganic phosphate formed (γ/ml.)	Per cent inhibition
None Alloxan (0.2%)	38	0	27	0
	10	74	19	30
None Zinc Sulfate (0.001 M)	92	0	49	0
	19	79	25	49

Since alloxan and zinc so markedly inhibit the formation of inorganic phosphate from glucose-1-phosphate and since the conversion of the 1-ester to the 6-ester is inhibited by them to an extent comparable to the degree of inhibition of the liberation of inorganic phosphate from glucose-1-phosphate,<sup>2</sup> it would appear as if the marked inhibition of zinc and alloxan in the formation of glucose from glucose-1-phosphate is, in large part at least, due to the suppression of the formation of glucose-6-phosphate. This would constitute additional evidence for the assumption that Cori ester is first converted to Robison ester prior to the dephosphorylation of hexosemonophosphate.

If it is assumed that glucose is derived from glucose-1-phosphate *via* two independent mechanisms, namely, directly from glucose-1-phosphate, and, indirectly after conversion of the latter to glucose-6-phos-

<sup>2</sup> Actually, slightly more inorganic phosphate is liberated than can be accounted for by the amount of glucose-6-phosphate apparently formed. Such figures are misleading. Alloxan must slow down the rate of formation of glucose-6-phosphate to the point where the rate of dephosphorylation of the latter exceeds the rate of its formation. Accordingly, at no time during the reaction can glucose-6-phosphate exist in appreciable quantities in the reaction mixture.



phate, alloxan and zinc might act both by inhibiting the direct dephosphorylation of Cori ester and by inhibiting conversion of the latter to glucose-6-phosphate. In such a case, it would be necessary to assume that the mechanism responsible for the dephosphorylation of glucose-1-phosphate is quite distinct from the one that obviously acts on the 6-ester. For alloxan is only moderately inhibitory towards the hexosemonophosphatase acting on glucose-6-phosphate (Table V) and, according to the hypothesis considered above, would have to be markedly inhibitory on an hypothetical enzyme responsible for the dephosphorylation of glucose-1-phosphate. Thus, even if it were proposed that glucose-1-phosphate could undergo direct dephosphorylation, it is essential to the facts to allow for the existence of a phosphatase capable of dephosphorylating the 6-ester but unable to act appreciably on the 1-ester.

### DISCUSSION

It is conceivable that the conversion of glucose-1-phosphate to glucose and inorganic phosphate may occur by one of the following four mechanisms: (a) the glucose may arise solely as the result of the direct dephosphorylation of glucose-1-phosphate by a phosphatase specifically active on this substrate only; (b) the glucose may be formed as a result of the dephosphorylation of both glucose-1-phosphate and glucose-6-phosphate by two distinct phosphatases one of which specifically acts on the 1-ester alone, the other of which is specific for the 6-ester; (c) the glucose may be formed as a result of the dephosphorylation of both glucose-1-phosphate and glucose-6-phosphate by a nonspecific phosphatase capable of effecting the dephosphorylation of both hexosemonophosphates; (d) the glucose may be formed solely as the result of the dephosphorylation of glucose-6-phosphate by a specific glucose-6-phosphatase after the conversion of glucose-1-phosphate to the 6-ester.

The data presented above are inconsistent with the first possibility considered. The course of the reaction is such that added glucose-1-phosphate is transformed rapidly to glucose-6-phosphate before liberation of the greater part of the inorganic phosphate. Obviously, at least the greater portion of the inorganic phosphate is formed as the result of the dephosphorylation of the 6-ester.

The possibility that glucose is formed as a result of the dephosphorylation of both glucose-1-phosphate and glucose-6-phosphate by two specific phosphatases is difficult to disprove. However, the good agreement between the curve in Fig. 1 calculated from the assumption that all of the inorganic phosphate is derived from the dephosphorylation of glucose-6-phosphate and the curve for the phosphoglucomutase reaction (8) fails to support such a possibility. If it were possible to obtain a preparation of liver hexosemonophosphatase free from phosphoglucomutase this question could be resolved, since it then could be determined whether glucose-1-phosphate could be dephosphorylated directly without first undergoing transmutation to glucose-6-phosphate. Future work on the possible separation on these systems is planned.

Even if it is postulated that liver extracts do contain a glucose-1-phosphatase, it is doubtful that such a system could contribute appreciably to the dephosphorylation of hexosemonophosphate. Under the conditions investigated, except in the presence of inhibitors, the velocity of the transformation of glucose-1-phosphate to hexose-6-phosphate was so much more rapid than the rate of dephosphorylation of hexosemonophosphate that, even when large amounts of Cori ester were added initially to the reaction mixture, the latter was practically all transformed to the 6-ester long before the process of dephosphorylation was well under way.

The possibility that the same phosphatase dephosphorylates both glucose phosphate esters appears to be denied by the effects of both zinc and alloxan. Both agents markedly inhibit the liberation of inorganic phosphate from Cori ester. Both agents, however, display a noticeably decreased inhibiting effect on the dephosphorylation of glucose-6-phosphate. Obviously, in view of these observations, the properties of an hypothetical glucose-1-phosphatase must be assumed to be quite distinct from those of the mechanism responsible for the dephosphorylation of glucose-6-phosphate, and one must deny the existence in liver extracts of a single mechanism capable of dephosphorylating both esters.

In summary, the following evidence favors adoption of the fourth possibility: Added glucose-1-phosphate is rapidly transformed to glucose-6-phosphate; the glucose-6-phosphate thus formed is then converted into inorganic phosphate and glucose; inhibitors that pre-

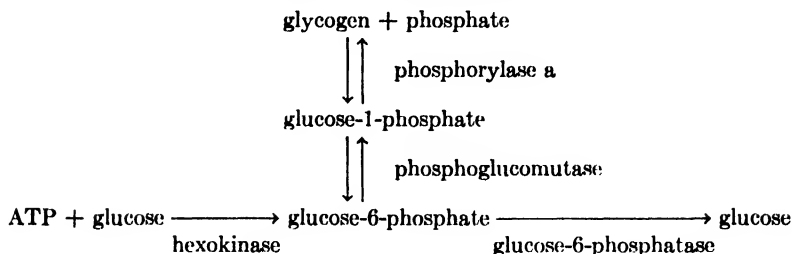
vent transformation of glucose-1-phosphate to hexose-6-phosphate also inhibit the formation of inorganic phosphate from Cori ester; agents which inhibit the dephosphorylation of glucose-6-phosphate also prevent the liberation of inorganic phosphate from glucose-1-phosphate. This evidence can be interpreted most clearly by the assumption that the transformation of glucose-1-phosphate to glucose-6-phosphate is an obligatory step in the glycogenolytic cycle. If this assumption is true, it is accordingly necessary to postulate existence in liver extracts of a single hexosemonophosphatase which acts specifically on the 6-ester only and which fails to promote dephosphorylation of glucose-1-phosphate. Such a degree of specificity is certainly not uncommon among the known phosphatases. It has already been demonstrated (2) that fructose-1-phosphate is probably not dephosphorylated by liver extracts without undergoing isomerization to glucose-6-phosphate and galactose-1-phosphate is not hydrolyzed by liver phosphatase (10). Accordingly, it would appear justifiable to designate the dephosphorylating mechanism investigated in this paper as "glucose-6-phosphatase."

Although no precise measurements can be reported at this time, preliminary observations indicate the maximum activity of this system to be in the region of pH 6.5. Whereas the role of the acid-base balance might be expected to vary with the choice of substrate, such observations, together with the other facts concerning the specificity of this system, militate against its confusion with the so-called acid and alkaline phosphatases also present in the liver.

Whether one can extend these observations gained from the use of extracts to the cycle as it occurs in the intact liver may be open to question. However, in view of the lack of any evidence to the contrary, and in view of the fact that the mechanism discussed above satisfactorily corresponds to the facts known about glycogenolysis, it seems justifiable to foster such an assumption. In accord with this step is the known fact that glucose-1-phosphate is never found in the intact liver. If, in the organ itself, as in extracts, the rate of the phosphoglucomutase reaction is so much more rapid than the process of dephosphorylation, the lack of accumulation of Cori ester would certainly tend to favor the hypothesis that, in the whole organ also, dephosphorylation proceeds *via* the glucose-6-phosphate stage.

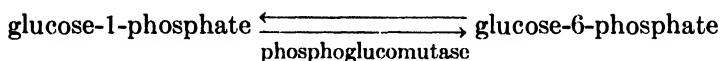
Accordingly, the mechanism involved in the regulation of the blood

sugar level by the liver may be depicted graphically as follows:

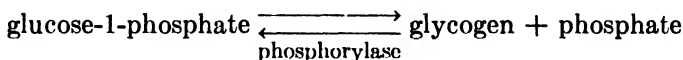


Such a scheme emphasizes anew the importance of glucose-6-phosphate in the carbohydrate metabolism of the liver, since it is the first intermediate formed in the conversion of glucose to glycogen and the last metabolite produced in the reconversion of glycogen to glucose. In other words, glucose-6-phosphate acts, as it were, as the fulcrum for both the glycogenolytic and the glycogenic mechanisms.

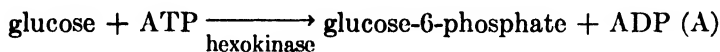
Since the reactions:



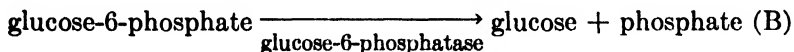
and



are reversible, any factor which inhibits the dephosphorylation of glucose-6-phosphate would tend to favor a retention of glycogen. Similarly, any factor which tends to prevent the formation of glucose-6-phosphate from glucose by the hexokinase reaction would tend to favor glycogenolysis. Thus, conditions which insure that the velocity of the hexokinase reaction (A):



surpasses that of the glucose-6-phosphatase reaction (B):



would tend to favor the formation of liver glycogen from blood glucose. Conversely, conditions which favor the velocity of reaction (B) over the velocity of reaction (A) would tend to favor the conversion of liver glycogen to blood glucose.

The demonstration of the effect of an increase in glucose concentration on the inhibition of the hexosemonophosphatase reaction corresponds well with what is known about the behavior of this cycle in the intact organism. Major and Mann (11) demonstrated that, in the presence of high concentrations of blood glucose, glycogen could be maintained in the liver of the completely depancreatized animal. Furthermore, we have demonstrated (12, 13) that high concentrations of blood sugar consequent to the intravenous injection of glucose, inhibit the ketogenesis and increased nitrogen metabolism of the diabetic animal. These phenomena may be satisfactorily explained in terms of the action of high concentrations of glucose on the glucose-6-phosphatase reaction. Since an increase in the concentration of glucose inhibits the dephosphorylation of hexosemonophosphate, extreme hyperglycemia would tend to favor retention of glycogen in the liver.

In view of the preceding it is obvious that the principal difference, in so far as the liver is concerned, between the diabetic and the non-diabetic organism is related to the relative velocities of the hexokinase and glucose-6-phosphatase reactions. In the diabetic organism, the velocity of the glucose-6-phosphatase reaction must surpass that of the hexokinase reaction even if the latter is not disturbed. In the non-diabetic animal, on the other hand, the velocity of the hexokinase reaction must surpass that of the glucose-6-phosphatase mechanism.

These considerations do not imply a direct action of insulin on either of the two systems. Thus, the observations of Colowick, Cori and Slein (14) clearly indicate no direct action of insulin on the hexokinase system and our own observations (15) confirm such a conclusion. Similarly, we have been unable to demonstrate a direct effect of insulin on the glucose-6-phosphatase system (16). In spite of this evidence that insulin has no *direct* effect on these two isolated systems, it is obvious from its action on the intact cell that it does affect these systems indirectly through its effect on some other mechanism.

### CONCLUSIONS

During the course of glycogenolysis in the liver, glucose-1-phosphate undergoes a preliminary conversion to glucose-6-phosphate prior to dephosphorylation.

The kinetics of these reactions and the influence of various inhibitors are described. The existence of a specific glucose-6-phosphatase system in the liver is demonstrated.

The role of the glucose-6-phosphatase system in the maintenance of liver glycogen is discussed.

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# Further Differentiation of Bean Trypsin-Inhibiting Factors<sup>1</sup>

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Received July 28, 1947

## INTRODUCTION

We have pointed out earlier that various types of beans such as navy (1), soy, white Chinese velvet, and Georgia velvet beans (2), contain trypsin-inhibiting fractions which are soluble in water but precipitate in alcohol. It is believed that such factors differ from the trypsin-inhibiting fraction of soy beans which requires acetone rather than alcohol for precipitation as observed by Ham and Sandstedt (3) and by the author (1).

Differences between this soy bean fraction precipitated with acetone and an alcohol-insoluble factor of soy beans have been noted (4). The primary purpose of the present report is to call attention to the further differences between this alcohol-insoluble soy bean factor, which has been crystallized by Kunitz (5, 6), and an alcohol-insoluble trypsin inhibitor found in relatively large amounts in navy beans. The differentiation also involves additional less prominent trypsin-inhibiting fractions of soy beans. In view of extensive work with bean trypsin inhibitors (7-14) these differences deserve emphasis.

## METHODS

The crystalline globulin factor of soy beans was prepared according to Kunitz' procedure (6) from ground untreated beans. The present preparations were found to have greater activity than earlier similar preparations obtained from treated commercial soy bean meal.

In preparing the navy bean trypsin inhibitor one liter of a 20% suspension of the ground untreated beans is allowed to stand several hours, preferably overnight. After

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<sup>1</sup> Read before the American Society of Biological Chemists, May, 1947. *Federation Proc.* 6, 241 (1947).



centrifuging, the supernatant solution is adjusted to pH 4. The precipitated proteins are centrifuged and discarded and 9 volumes of alcohol is added to the supernatant solution. After vigorous stirring, the precipitate, which rapidly settles out, is centrifuged, and dissolved in 100 ml. of water. To this is added 100 ml. of 5% trichloroacetic acid. After heating at 80–85°C. for 5 minutes the resulting precipitate is centrifuged and discarded. The supernatant solution is adjusted to pH 9 and sufficient ethyl alcohol is added to provide a concentration of 60%. After centrifuging and discarding the precipitate, additional alcohol is added to the supernatant solution to bring the concentration to 85%. The final precipitate is centrifuged and dried in a vacuum desiccator.

Such preparations can be further concentrated by discarding the precipitate which forms upon half saturating dilute solutions with ammonium sulfate and retaining the precipitate obtained by increasing the salt concentration to 0.7 saturation. The final precipitate is dissolved in water, dialyzed,<sup>2</sup> reprecipitated with nine volumes of alcohol and dried. Typical yields vary from 450 to 550 mg.

The second inhibitor of soy beans, which appears to be quite similar to the navy bean factor, can be prepared in much smaller amounts by the procedure applied to navy beans. To eliminate other trypsin inhibitors the procedure was repeated, employing smaller volumes, when applied to soy beans. The amount of material obtained may vary between 40 and 50 mg.

A third inhibitor of soy beans has been found in very small amounts associated with the crude acetone-insoluble fraction which was described earlier (4). One liter of a 20% suspension of ground untreated soy beans is shaken in 60% ethyl alcohol for 15 minutes. The centrifuged and filtered extract is precipitated with 2 volumes of acetone. After freeing the precipitate of acetone by evacuation, it is dissolved in 40 ml. of water to which is added 40 ml. of 20% trichloroacetic acid. After centrifuging, 10 volumes of acetone is added to the supernatant solution. The resulting precipitate is collected by centrifuging. The supernatant solution is discarded and the remaining acetone is removed from the precipitate by reduced pressure. The precipitate is dissolved in 60 ml. of water to which is added 40 ml. of saturated ammonium sulfate. The resulting precipitate is discarded. The concentration of ammonium sulfate is then increased to 0.5 saturation and this precipitate is centrifuged, redissolved in water, dialyzed, reprecipitated with acetone and dried. Yields have averaged about 75 mg.

The degree of inhibition of tryptic digestion of casein was estimated as described earlier (4).

## RESULTS

Precipitation of the various factors with ammonium sulfate was followed by comparing the inhibiting capacity of the material precipitated from a 0.1% solution of a weighed amount of a given inhibitor with that of an equal weight of the untreated fraction. All material which precipitated in the salt solution was centrifuged down and re-

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<sup>2</sup> 27/32 Nu Jax Visking Cellulose Sausage Casing, The Visking Corporation, Chicago, Ill.

dissolved in water. Its ability to retard the digestion of casein by trypsin was determined in triplicate simultaneously with a similar determination employing the untreated material. The data of Table I which are typical of numerous observations indicate more objectively

TABLE I  
*Comparison of Trypsin-Inhibiting Factors*

Treatment and portion used	Per cent of normal uninhibited digestion in the presence of inhibiting factors			
	Crystalline soy bean factor 0.004% <sup>a</sup>	Navy bean factor 0.004% <sup>a</sup>	Second soy bean factor 0.004% <sup>a</sup>	Third soy bean factor 0.02% <sup>a</sup>
Untreated	43.0	31.0	48.2	50.7
Material insoluble in 0.4 saturated (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	45.5	98.5	99.0	98.5
Untreated		34.0	49.5	51.0
Material insoluble in 0.5 saturated (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>		99.0	96.2	53.2
Untreated		35.3	50.8	
Material insoluble in 0.7 saturated (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>		37.5	52.0	
Untreated	45.0	32.8	48.5	52.0
Material soluble in 2.5% CCl <sub>3</sub> COOH	97.0	34.5	51.9	54.0
Untreated		32.5	49.3	50.7
Material soluble in 10% CCl <sub>3</sub> COOH		102.0	98.0	51.0

<sup>a</sup> The concentration of the untreated inhibiting fraction in the final casein digestion mixtures. That of the treated material would have been the same except for the influence of the treatment.

what is apparent in preparing and dealing with these factors. It is observed that the crystalline globulin factor precipitates in 0.4 saturated ammonium sulfate. The navy bean factor and the second soy bean factor require a greater concentration of the salt, such as 0.7 saturation. The third soy bean factor precipitates at 0.5 saturation.

Similarly, the behavior of the various preparations in 0.1% concentration in trichloroacetic acid was observed. The material which

remained in solution in the acid was precipitated with alcohol or acetone and the inhibiting capacity of this precipitate was determined. Acetone was used only with the third soy bean factor. From the tabulated data it is observed that the navy bean factor can be almost quantitatively recovered from the supernatant solution of 2.5% trichloroacetic acid. The crystalline globulin factor is precipitated from such a solution (5, 4). The navy bean inhibitor and the second soy bean factor precipitate in 10% trichloroacetic acid but the third soy bean inhibitor is soluble at this concentration of the acid.

In our experience the crude navy bean preparations have been found to be considerably more active than the most active crystalline globulin soy bean preparations obtained from untreated beans. The navy bean factor is precipitated with picric and tannic acids but fails to give a positive test with the nitric acid ring test or with potassium ferrocyanide in acid solution. The biuret and Millon's tests are positive. In contrast with the globulin factor it is quite soluble in pure water. The activity and solubility characteristics of the navy bean factor suggest that it has a smaller molecular weight, more nearly resembling plasma trypsin inhibitor in this respect.

The third soy bean inhibitor is much less soluble in alcohol than is the first acetone precipitate from which it is obtained. However, the active material is not precipitated by alcohol from dilute solutions (such as 0.1%, as used in much of the present work). Furthermore, it is precipitated from such dilute solutions with acetone only in the presence of a small amount of a salt such as sodium chloride in a concentration of 0.25 mg./100 ml.

All of the foregoing factors appear to differ from the typical acetone-insoluble fraction of soy beans. Further work with this fraction which is in progress will be described later; however, it may be noted that, in contrast with the above fractions, this factor readily dialyzes and is not adsorbed on 5% kaolin. It is precipitated from the dialyzate with acetone but not with alcohol. These properties correspond with those of the fraction described earlier by Ham and Sandstedt (3). Soy beans contain large amounts; however, a low order of activity prevails in the present preparations which contain relatively large amounts of phosphorus.

#### ACKNOWLEDGMENT

The author wishes gratefully to acknowledge the technical assistance of Miss Gwendolyn Brock.

## SUMMARY

A highly active trypsin inhibitor which can be readily obtained in relatively large amounts from navy beans differs from the crystallized globulin factor of soy beans. Soy beans also contain other less prominent trypsin-inhibiting fractions.

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# **Zoopherin: A Nutritional Factor for Rats Associated with Animal Protein Sources<sup>1</sup>**

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*Received August 4, 1947*

This report deals with a condition induced by yeast-containing plant rations which, when fed to sexually mature female rats, leads to normal frequency of pregnancies without resorptions or lactation failure and still induces a high mortality in the young. This occurs after lactation has been completed.

The missing factor is found associated with animal protein sources and is certainly allied to what has been designated as "animal protein factor" in recent poultry literature. It is water-soluble and is, therefore, not identical with the petroleum ether-soluble (1) or ether-soluble factor (2). Until it can be fitted into a reasonable nomenclature and to avoid repetitious circumlocution, it may be called "zoopherin," a factor needed to carry on animal species. It should not be classed among the B factors if the historical convention is followed of restricting this term to water-soluble substances found in yeast.

It may be identical with the factor Rubin and Bird (3) prepared from cow manure which affects hatchability and survival of chicks on plant rations. On diets containing an elaborately purified casein, Cary, Hartman and Dryden (4) have produced a deficiency state in rats very similar to that described below. The results with liver and pancreas on growth and nitrogen utilization in mice reported by Bosshardt *et al.* (5), may also be attributable to the same factor.

## **PRODUCTION AND NATURE OF THE DEFICIENCY**

To give a clear demonstration of the uncomplicated deficiency signs all other nutritional factors should be adequate. On the basis of many

<sup>1</sup> This work was reported in part before the Am. Chem. Soc. at the 110th and 111th meetings in 1946 and 1947, respectively.

TABLE I  
*Diet Composition<sup>a</sup>*

	Pr 60	Pr P	Pr 30	1551
Cottonseed meal ("Proflo")	60.	30.	30.	
Crude casein		12.5		46.4
Ground yellow corn			26.5	
Wheatsthworth whole wheat flour			27.	
Cerelose	14.8	34.3		19.4
Brewer's yeast extract <sup>b</sup>	2.0	2.0	0.5	
Alfalfa leaf meal	5.0	5.0		
CellufLOUR carrying B and K supplement <sup>c</sup>	2.0			22.0
Bone meal <sup>d</sup>			10.0	
Gelatin	4.0	4.0		
Bone ash	6.0	6.0		6.0
Iodized salt			1.0	
Mod. Wesson salts (Ca and P free)	1.2	1.2		1.2
Vitamin K			166 $\gamma$	
Carotene concentrate in oil <sup>e</sup>	0.1	0.1	0.1	0.1
Viobin wheat germ oil	0.25	0.25		0.25
Cod liver oil	1.0	1.0	0.25	1.0
Cottonseed oil	3.65	3.65	4.65	3.65

<sup>a</sup> Diets Soy 60 and Et 60 were identical with Pr 60 except for the substitution of Staley Lo-Fat soybean meal and ethyl ether-extracted raw cottonseed, respectively, for Proflo.

<sup>b</sup> Type 3, Standard Brands.

<sup>c</sup> Provides per 100 g. diet, 1 mg. each of thiamine and pyridoxine hydrochlorides, 2 mg. riboflavin, 4 mg. each of calcium pantothenate and nicotinic acid, 200 mg. choline chloride, and 0.5 mg. vitamin K<sub>2</sub> (2-methyl-1,4-naphthoquinone).

<sup>d</sup> At first, 10% bone meal was used in all these diets, but when it became unavailable during the war we substituted bone ash and gelatin. More of the work on Pr 60 and Pr P has been done with the substitute.

<sup>e</sup> Contains 7,500 units A activity/g.

control observations the diets used are well fortified and undoubtedly over-fortified in protein and the known vitamin factors. The principal protein sources which we used were a hydraulic press cottonseed flour which is made under very well controlled conditions of moist heat treatment, and a heat-treated soybean meal. Diet compositions are given in Table I.

The cottonseed flour which was used (data on this are given in a previous report (10)) goes under the name of Proflo and proved to be a remarkably reproducible and stable die material as compared with other plant protein sources.

Various lots of crude casein which have been used were of rather uniform quality as active supplements. However one lot had very low activity and another became inactive during two years storage at room temperature.

The only liver preparation used in this work was Wilson 1:20 liver powder.

If stock diet female breeders were put on diet Pr 60, there was prompt impregnation when mated, there was no difficulty during gestation (no resorptions), and examination of the newborn indicated good lactation. Growth of the young continued normally for about 3 weeks, after which there was a slight falling off of the growth rate, and around 30 or 31 days of age very high mortality set in. At weaning (28 days) the weights were about 15% low. Of 66 rats, mostly negative controls in the curative experiments to be described further on and which were continued on diet Pr 60, all grew poorly and 45% died, usually within a few days of weaning. Survivors continued to grow slowly with no further crises and were 40% below their normal weight at 20 weeks of age. There was a tendency for the litters either to survive entirely or wholly succumb, thus indicating differences in storage, requirement, or possibly synthesis of the missing factor. This is similar to the recently reported experience of Rubin and Bird (6) with chickens on plant rations.

The probable cause of death at the time of the crisis is hemorrhage in the fundic portion of the stomach. We have made reference to these findings in a report on nutritional effects on the gastric mucosa (7). No obvious lesions were seen in any other part of the gastrointestinal tract, although the small intestine appears to show throughout its length a rather atrophic condition with thin, almost transparent walls, and, unless autopsies are performed promptly, enough autolysis may take place so that intestinal contents are found in the peritoneal cavity.

The blood of such animals showed high urea values. The rat's blood urea varies considerably with protein intake and the values recorded were high when compared with normal animals of similar nitrogen intake (see Table II). Whether the urea changes are referable to altered intermediary nitrogen metabolism or due to changes in kidney function or other causes is not known. At the time of the crisis, there is a very definite leukopenia (see Table III). There also appears to be a low blood volume and the spleen is remarkably shrunken.

There are a number of other changes which, however, require further study before they can be rationally fitted into the zoopherin deficiency



syndrome or definitely excluded. On diet Pr 60 the offspring consistently show enlarged kidneys, averaging nearly 50% overweight according to the standards for this organ (8). These kidneys show a normal water content (no edema), and histologically no signs of abnormal structure are seen. Thus, they differ from the kidney condition described for choline deficiency. In any case the diet contained ample choline. A liver enlargement almost parallels the kidney hypertrophy. Sections show no excess fat in the liver.

Since animals from stock diet mothers when fed diet Pr 60 from weaning time to adult age at no time show the kidney hypertrophy, it seems likely that the change which does occur in the young whose

TABLE II  
*Effect of Zooperin Deficiency on Blood Urea*

	Urea N	NPN blood <sup>a</sup>		Urea N	NPN blood
Pr 60—1st generation	<i>mg./100 g.</i> 27.0(8) <sup>b</sup>	52.1(8)	Stock 8-300d. (indep. of age) <sup>c</sup>	<i>mg./100 g.</i> 17.2(27) ±.55	42.1(23) ±.4
Pr 60 offspring 14 d.		42.8(8)			
27-29	24.4(5)	49.9(5)			
31-35	69 (13)	100 (17)	Effect of dietary N		
41-56	31.8(28)	57.3(26)	5% casein-56 d.	13.1(6)	36.1(6)
134-138	27.5(6)	53.4(6)	27% casein-49 d.	24.5(6)	47.5(7)
Prevention <sup>d</sup>			46% casein-84 d. (diet 1551, Table I)	33.0(2)	61.5(7)
Offspring on Pr 60+liver or crude casein					
31-35 d.	26.2(6)	54.0(4)			
41-56 d.	26.4(11)	52.3(10)			

<sup>a</sup> Heart blood obtained at autopsy. Rats were killed with ether. Analyses were made on the tungstate filtrate prepared by running whole blood into a weighed known volume of the Van Slyke and Hawkins combined reagent (Peters and Van Slyke, *Quantitative Clinical Chemistry*, Ed. 1, Vol. I, p. 66). No animals were included which showed hydronephrosis.

<sup>b</sup> Figure in parenthesis is the number of rats averaged.

<sup>c</sup> Urea N on females averages 1 mg. lower than on males. Standard errors on the various other means run from 1.5 to 3, except for the 31-35 day old Pr 60 offspring, where the standard errors are about 6.

<sup>d</sup> Presumably because of the high N level in Pr 60 we are unable to get Urea N's as low as on stock. Pr 60 first generation, Pr 60 offspring with a preventive supplement, and old surviving Pr 60 offspring all come to about the same level of urea N and NPN.

TABLE III  
*Effect of Zoopherin Deficiency on White Cell Count*  
 (Number of cells/mg. blood) <sup>a</sup>

Age	Offspring on diet Pr 60		Stock diet
	Deficiency	Prev. by liver	
27-29 d.	6,470(2) <sup>b</sup>		7,750(5)
31-35	1,810(7) <sup>c</sup>	9,020(4)	8,400(8)
41-56	9,370(15)	10,280(10)	10,200(12)
134-138	13,000(4)		

<sup>a</sup> Heart blood obtained at autopsy. Rats were killed with ether.

<sup>b</sup> Figure in parenthesis is the number of rats averaged.

<sup>c</sup> Of these 7 rats, the 3 with lowest counts, including the extraordinarily low figure of 570, had been bred and raised on Pr 60 + 10% dried brewers' yeast. The leukopenia dissociates itself from folic acid or any other yeast factor deficiency.

mothers were on the deficient diet is due to interference with one or the other of the postnatal developmental processes in this organ such as discussed by Cutting and McCance (9).

An additional kidney symptom makes its appearance at about seven weeks of age; namely, hydronephrosis. Its incidence is quite variable, occurring in only about 10-15% of the animals.

In the preventive studies described further on, it was found that the supplements which effectively prevent failure of survival or growth do not always prevent hypertrophy, although they reduce it. It appears, therefore, that, for normal kidney and liver development, either much larger preventive supplements are needed or else another factor is concerned with these conditions. Hydronephrosis definitely does not appear inversely to growth and survival. It seems to be prevented by crude casein.

The same typical picture of a postweaning crisis was obtained on diets containing ether-extracted cottonseed instead of the hydraulic press cottonseed meal. On the corresponding soybean meal diet, there was, in addition, some indication of poor lactation.

#### PREVENTIVE STUDIES

Crude casein, introduced into diet Pr 60 (12.5 parts casein plus 87.5 parts Pr 60) and fed to females from impregnation time on, completely prevented the symptoms in the young and growth was excellent. This experiment was not carried beyond one

generation of offspring, but a diet of similar N content as Pr 60 containing 12.5% crude casein and 30% of "Proflo" (diet Pr P, Table I) when fed continuously produced exemplary rats through successive generations (see Fig. 2, left), comprising 12 litters of the second and 12 litters of the third generation.

Five per cent of liver extract powder (Wilson 1:20) added to diet Pr 60 also led to good growth and survival. One-half per cent of liver powder was not quite as effective.

Successful preventive experiments with good growth and survival were also carried out with "fish solubles" (condensed fish press water—Borden) at levels of 5 and 10% (2.5 and 5%, respectively, of solids).

The addition of 10% of dried brewers' yeast (two different lots of Standard Brands type 2019 and one lot of Anheuser Busch type K) was totally ineffective.

### *First Generation Effects and Protein Level*

Experiments beginning at the time of impregnation are suitable for isolating the principal symptoms; namely, the crisis appearing at the end of a 4-weeks' lactation period. They also led to the working out of a technique for curative tests. When, however, rats are put on the deficient diet at 4 weeks of age and continued on this regime through a pregnancy, the deficiency is apparently more marked and not infrequently there will be some deaths among the offspring during the early days after parturition. This may be due to faulty lactation; there is, however, no interference with fertility or gestation.

Another factor which should be considered is the protein level of the diet. This can be demonstrated by body weight data even in first generation experiments, *i.e.*, with weanling rats whose mothers were on stock diet throughout. Four-week old rats put on a diet of 20% cottonseed flour or soybean meal and white flour, having a nitrogen content of about 3.6%, will grow optimally to the age of 14 weeks, at which time the experiment was discontinued (10). With a diet of cottonseed meal, whole wheat and corn, fortified with sources of known essentials and having a nitrogen content of 4.3%, growth began to slow up at 11 weeks of age. (Typical of a number of such diets is Pr 30 in Table I.) With Pr 60 (N = 6.5%) growth retardation began at 6-7 weeks of age (see Fig. 1, left, and Fig. 2, right). Female rats on this diet were 15% under weight at 20 weeks of age. When now given a source of zoopherin, their growth promptly speeded up to return to the optimal growth curve in 11 weeks (see Fig. 2, right). Stock weanling rats placed on a normal diet based on crude casein and sugar designed to have the same nitrogen content, caloric density, and digestibility as Pr 60 (diet 1551, Table I), grew normally for 12 weeks.

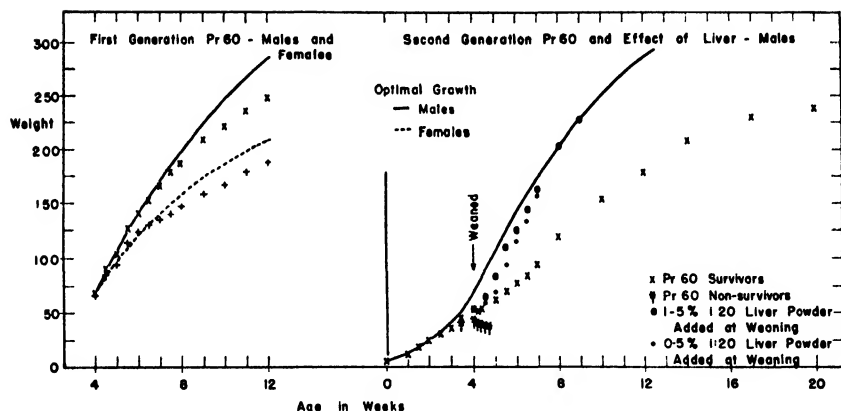


FIG. 1. First and second generation growth on Pr 60, and the curative effect of a liver supplement.

Left hand graph: 4-week old stock weanling rats were placed on diet Pr 60. Right hand graph: The rats were the offspring of stock diet females which had been placed on diet Pr 60 at time of impregnation. The young were weaned at 4 weeks, when liver supplementation was started. Many of the animals without supplement died.

It may be noted that the effect on the first generation growth in zoopherin deficiency becomes more marked as the experiment proceeds, with an increasing percentage difference compared to controls (Fig. 1 and Fig. 2, right). This applies to young and old animals and at the various protein levels. It is easily distinguished from protein deficiency, where the most marked effects are shown early.

The interplay of protein level and a liver factor has also been illustrated by Bosshardt *et al.* (5), in mice with diets containing 20 and 35% purified casein, the latter level being above the optimum for mice. In the experiments of McIntire *et al.* (11), and Sporn *et al.* (12), with weanling male rats on the suboptimal level of 18% casein, the growth curve (13) is typical for moderate protein deficiency, the rats recovering spontaneously from the more marked early deficiency. Compared with controls, the growth deficit is greater at 2 weeks than later on (12). Under such conditions, activity on the part of amino acid-containing supplements (milk, liver powder, *etc.*) does not afford cogent evidence for a new factor (11, 12) nor is it necessary to link the spontaneous recovery to intestinal synthesis (12).

*Addendum:* Since this was written a report by Jaffe and Elvehjem appeared in *J. Biol. Chem.* 169, 287 (1947), in which a liver factor thought to be heat-stable leads to increased growth in rats on all-plant rations and also to a lesser extent on diets containing "purified casein." Although the effects are small and not any too consistent by the technique which they used, the findings are similar to the zoopherin

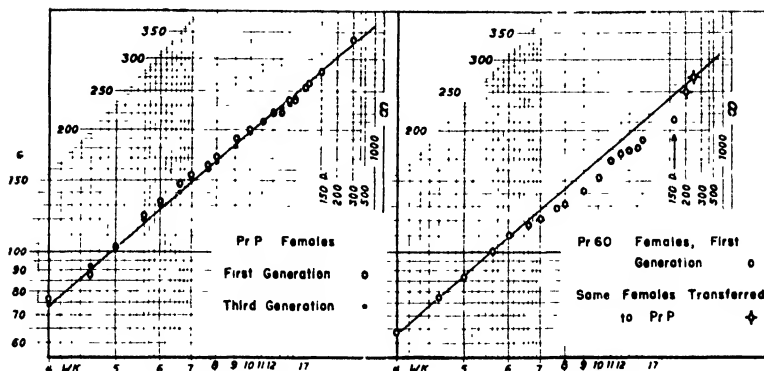


FIG. 2. Preventive and curative effect of crude casein.

The left hand graph is a log weight reciprocal age plot of the growth in 12 females of the first generation and 11 females of the 3rd generation on diet Pr P, in which 12.5% crude casein has been substituted for some of the cottonseed flour. The experiment was discontinued when the 3rd generation reached 15 weeks. Growth was optimal throughout (straight line of slope  $2.84 \frac{\log \text{ weight in gs.}}{\text{reciprocal weeks}}$  for females, intercept varies with the mean body size or stature of the particular group of rats). There was no deterioration in average size. Optimal male growth was also obtained.

The right hand graph is a similar plot of the first generation growth in 8 females on Pr 60 who were changed to the crude casein diet Pr P at 23 weeks, after the weaning of 1 litter. The growth on the unsupplemented diet deviates from the optimal curve in the same way as the left hand data of Fig. 1 obtained 3 years later. The females were bred again on the supplemented diet; the average 4-week weight of the litters on the deficient diet was 46; on the supplemented diet 64.

action we have described. It does not appear from the record whether or not the growth deficit is greater at two weeks than later on, as was emphasized in the paper of Sporn *et al.*

## CURATIVE STUDIES

### *Preliminary Trials*

Of 19 male survivors of a number of litters from dams which had been on diet Pr 60 from weaning time on, 3 experimental groups were made up. They had proceeded with very slow growth to ages of 42–55 days. One group was transferred to stock diet, the second was continued on Pr 60 but with a supplement of 5% liver extract powder, and a third group continued on diet Pr 60 with a 10% Labco casein (vitamin-free) supplement (Fig. 3). The first two groups showed spectacular recovery while the purified casein supplement led to no increase in growth. After 17 days, some of the animals on Labco casein were switched to a crude casein supplement, whereupon their growth picked up sharply (Fig. 3). It appears that the unknown factor is present in

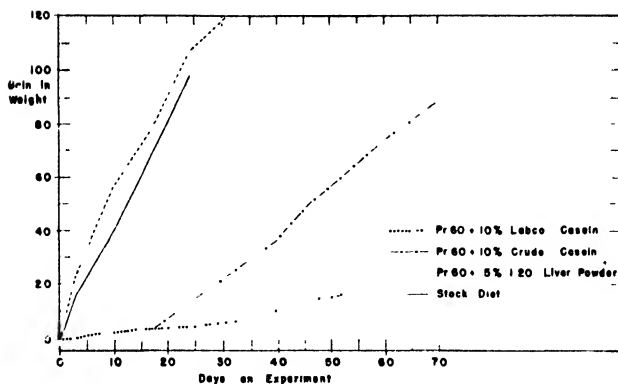


FIG. 3. Preliminary curative trials.

Nineteen very severely affected male survivors from mothers who had been on Pr 60 since they were weaned were divided into the 3 groups indicated. The experiment began when the rats were 42 to 55 days old (see text p. 122).

ample amounts in stock diet which contains dried milk and meat scrap, that liver extract and crude casein represent good sources and that the sample of purified casein used is not a good source. Furthermore, the experiment shows that rapid growth recovery is possible even at this late date.

While the principal signs of the deficiency are shown in the offspring, the mother rats also do not fare too well, judging by body weight and general behavior. Recovery of mothers was demonstrated by shifting some mothers which had had one litter on diet Pr 60 to a diet (Pr P of Table I) with a crude casein supplement, and breeding again (Fig. 2, right). The offspring of the second litters survived, and showed in every way a normal behavior, with body weights throughout as good as those on stock diet. Continued breeding on diet Pr 60 leads to rapid deterioration of the young in successive litters.

#### *Plan for Testing Supplements by Curative Technique*

To carry out curative tests, it became necessary to find some means of reversing the temporary handicap which occurred in each breeding cycle, in a reproducible manner. Good results were obtained if breeders from the stock colony were placed on diet Pr 60 at the time of successful impregnation and if, after weaning the litters, the dams were given a three weeks' rest period on stock diet. Under this plan, the breeders remained in good condition and in the offspring there was no noticeable deterioration, even in the fourth litter as compared with the first.

It has become quite evident that the variability in behavior of the offspring used for testing is less within the litter than between litters

and the results are more constant for the individual dam. For this reason, successive litters from tried animals are desirable.

Administration of supplements for curative tests was begun at the age of 28 days on the offspring of rats on ration Pr 60 from impregnation time on. All litters were reduced to 6, preferably of the same sex, within 4 days after birth. One positive control (supplement of 5% of 1:20 liver powder) and at least one negative control were taken from each litter. Matching, with regard to sex and litter origin, is essential; matching with regard to body weight was carried out as far as possible. Observations were usually continued for 3 weeks; *i.e.*, to 7 weeks of age. Judgment was on the basis of growth and survival.

A series of levels of liver extract powder was studied and some of the results are given in Fig. 1, right. One per cent, or higher, levels led to maximal recovery rates and there were no deaths. (The 5% level used in the preventive experiments is probably unnecessarily high.) The animals soon reached their normal growth curve, females sooner than males because they did not have so far to go. A 0.5% level appears somewhat less effective but is still quite active. This corresponds to the partial effect in the preventive studies at this level. A supplement of whole dried liver from 7-week old rats which had served as negative controls in curative tests was entirely inactive when fed at a level of 2.5%, while livers of 7-week old rats from dams which had received a preventive supplement (fish solubles) on diet Pr 60 were active, although not maximally, at this level.

Two other preparations have been found to be active—commercial fish solubles (Borden), and a concentrate made from cow manure by Rubin and Bird. Fig. 4 shows the results of curative tests on these materials at various levels. Under these same experimental conditions, negative results on growth recovery accompanied by other symptoms were obtained with the following materials: Labco lactalbumin (10%), Borden's milk vitamin concentrate (5%), dried brewers' yeast (10%), rice bran extract (5%).

Because of the possibility of methionine or lysine deficiency in heat-treated vegetable proteins, supplements of 0.2% DL-methionine and 1% L-lysine were tried, but were found ineffective.

Since yeast and yeast extract (already present in diet Pr 60) are ineffective, and since both are good sources of folic acid activity in the rat treated with sulfa drugs, it seemed unlikely that folic acid could be the missing factor. However, we tried a concentrate of Bc from liver at levels of 9.25 and 18.5  $\gamma$  of Bc per day; total administration was 296  $\gamma$  per rat at the higher level and half that amount at the lower level. At the lower level, which, by existing criteria, affords ample folic acid, the result was plainly negative. At the higher level, the animals given the supplement showed a barely detectable superiority in growth over the controls. Therefore, Bc or folic acid is not the missing factor but this factor may accompany Bc to a certain extent during purification.

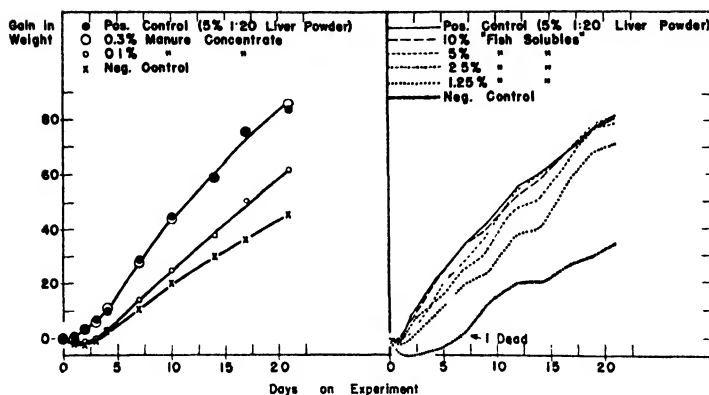


FIG. 4. Curative assays on manure concentrate and fish liquor.

In the fish liquor assays 3 litters were used, two of which were female, 1 male. In the manure concentrate assay there are 4 females and 2 males on each experimental level, 2 females and 1 male in each control group.

### *Preliminary Characterization of Zoopherin*

The material which is active in curative tests, using either liver or fish solubles as a source, is very soluble in water, dilute acid<sup>2</sup> and alkali, and dilute alcohol, moderately soluble in 95% alcohol and insoluble in petroleum ether and ethyl ether. Unless it can occur as a lipid-soluble compound or conjugate, it is, therefore, not the same as the lipid-soluble "animal protein factor" referred to by Heuser *et al.* (1) and by Johnson *et al.* (2). It is relatively quite stable to heat, exposure to light and air, dilute acid or alkali and activity is not lost during concentration procedures. It is subject to salting out with  $(\text{NH}_4)_2\text{SO}_4$ . It is not precipitated by basic lead acetate (10% solution of lead subacetate Merck, pH 7.5). It is not precipitated by trichloroacetic acid or picric acid.

### DISCUSSION

It is probable that 5 conditions affect the response of animals to the deficiency. One is obviously the level of the missing factor in the

<sup>2</sup> The concentrate of Rubin and Bird was precipitated at pH 3. A further purification step introduced by Dr. Bird was digestion with papain after which the active material was soluble in both acid and alkaline aqueous solution. The material we received from Dr. Bird was the earlier preparation.



experimental diet, (2) is the level of nitrogen in the experimental diet, (3) is variation in susceptibility, (4) is the status of bodily stores and (5) is the amount of the factor in the maternal diet. There is probably considerable overlapping between (3) and (4), and (4) and (5). However, the last two conditions (4 and 5) are not equivalent, since there is a further very important point connected with the maternal diet; unless this is deficient, there is no kidney hypertrophy and none of the critical phenomena in the young. Apparently there is a special requirement for zoopherin during early postnatal life, and, if the animal gets safely past this stage, a deficiency, induced or persistent, at any later time is much less severe and of a different type.

The existence of bodily stores which are rather slowly depleted can be demonstrated without the complicating effect of this specialized early requirement in the offspring by the pronounced deterioration in quality of successive litters on Pr 60 when the mother is not rested on stock diet between litters (depletion of maternal stores) and by the poorer quality of even first litters on Pr 60 if the mother has been on the diet from weaning instead of just during gestation and lactation. Only after such severe depletion of maternal stores is there interference with normal lactation. This response may be contrasted with the response to a purified diet with no added folic acid, where there is immediate interference with lactation, the effect being almost as great if the experimental diet is started at the birth of the litter as when the mother had been on the diet since she was weaned (14). Further evidence of slow depletion of bodily stores is furnished by the fact that stock weanling rats placed on Pr 60 grow just as rapidly for the first 2-3 weeks as the offspring of dams given a supplement of zoopherin. To demonstrate and work with zoopherin deficiency it is essential that all factors be provided in ample amount whose deficiency produces a more immediate effect—such as amino acids, folic acid (for lactation in rats and growth in chicks), and various other possible factors found in yeast needed for growth in chicks (15, 16) and lactation in rats (14).

The effect of the maternal diet on the response of the young is probably a combination of an effect on bodily stores of the young and the establishment of a deficiency during a period of specialized requirement. Thus, young rats on Pr 60 behave very differently according to whether the maternal diet is well supplied with zoopherin (stock diet) or deficient (Pr 60). It is quite probable that a stock diet with a less adequate supply of zoopherin would produce weanling animals which

respond more markedly to a deficient diet (larger apparent first generation effect). The results on chicks show that the quality of the hen diet determines whether chicks on a deficient diet grow normally or poorly in the first 6 weeks after hatching, and that chicks from commercial hatcheries are not always fully protected (3, 17).

A consideration of related work reported by others leads to the following tentative estimate of the situation. In a number of cases improved growth or improved lactation has been observed after administration of liver preparations. Frequently, improvement also resulted from the administration of yeast or other sources of factors contained in yeast. In this category, as far as rats are concerned, belong the observations of Spitzer and Phillips (18) on all plant rations and those of Nelson and Evans (14) on purified diets. Cerecedo and co-workers (19) had similar observations on mice as well as rats. The extensive work of the Cornell group (20) is of particular interest because they demonstrated this point in chicks. The effect of animal protein supplements is brought out clearly only after the factors R and S from yeast are supplied.

As mentioned above, the observations of Rubin and Bird (3) probably deal with a factor identical to zoopherin. In the case of Cary *et al.*'s (4) rat experiments this is also indicated. The factor associated with animal proteins and absent from plant sources is removed from casein by Cary *et al.* We confirm Cary's judgment that commercially purified caseins are free from the factor to a variable extent. Incidentally, on the highly purified casein rations, kidney hypertrophy was found (personal communication from Dr. Cary). In the mouse studies of Bosshardt and collaborators (5), only first generation experiments were performed and, therefore, the most striking symptoms described above were not recorded. However, the general character of the work and especially the intensification of the deficiency state with increased protein indicates that their factor from liver and pancreas is probably also zoopherin. Such effects of high protein levels have also been noted by Cary and by Bird. The most significant agreement between the observations reported here and those of the three last mentioned authors can be seen in the fact that the factor concerned is not found in yeast.

Zoopherin can also be distinguished from a number of other postulated, or only partially characterized, factors on the basis of its distribution. Thus, we can say that it is not the same as the strepogenin of Woolley (Scott, Norris and Heuser (21) having found yeast a good source of strepogenin), factors R and S of Norris and co-workers (15), B<sub>10</sub> and B<sub>11</sub> of Elvehjem and co-workers (16), and U of Stokstad (22). While neither zoopherin nor the monkey factor of Elvehjem and co-

workers (23) is present in yeast, the two are distinguished by the absence (24) of the latter from 1:20 liver powder, fish liquor and Bird's cow manure concentrate. Zoopherin is relatively stable, while the monkey factor, which occurs in whole liver, raw meat and milk, is readily destroyed by heat. Zoopherin also appears to differ from the factor for cats reported by Poling (25) and co-workers, and the heat-labile factor for foxes of Schaefer, Whitehair and Elvehjem (26), which, in distribution and in lack of stability, resemble the monkey factor.

#### ACKNOWLEDGMENTS

Thanks are due to investigators for gifts of materials: To Dr. H. R. Bird, for the chick growth factor; to Dr. A. A. Altschul and the Southern Regional Research Laboratory, U. S. Dept. Agr., for the ether-extracted cottonseed; to Dr. J. J. Piffner for the Bc concentrate.

We also gratefully acknowledge the liberal supply of "Proflo" from the Traders Oil Mill Co., Fort Worth 1, Texas, the "fish solubles" from the Borden Co., and the "Lo-Fat soy meal" from the A. E. Staley Co., Decatur, Ill.

#### SUMMARY AND CONCLUSIONS

1. For successful propagation, the rat requires another factor besides the known essentials which are required for fertilization, gestation, and lactation. Zoopherin is suggested as a suitable, tentative name.

2. The deficiency reveals itself most strikingly after the natural lactation period by a marked growth restraint, high mortality, high blood urea, and low white cell count.

3. Zoopherin has so far been characterized chemically only by its solubilities and by fractional precipitations. It may be of polypeptide nature and is relatively stable.

4. Zoopherin, as far as characterized by its deficiency signs, seems to be identical with the "nutritional factor X" of Cary and Hartman and with the chick-growth factor from cow manure of Rubin and Bird. The chick growth factor relieves completely the symptoms of zoopherin deficiency in rats. It has the properties of one of the substances designated as "animal protein factor" in the poultry literature.

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# A Microchemical Reaction Resulting in the Staining of Polysaccharide Structures in Fixed Tissue Preparations

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Received August 11, 1947

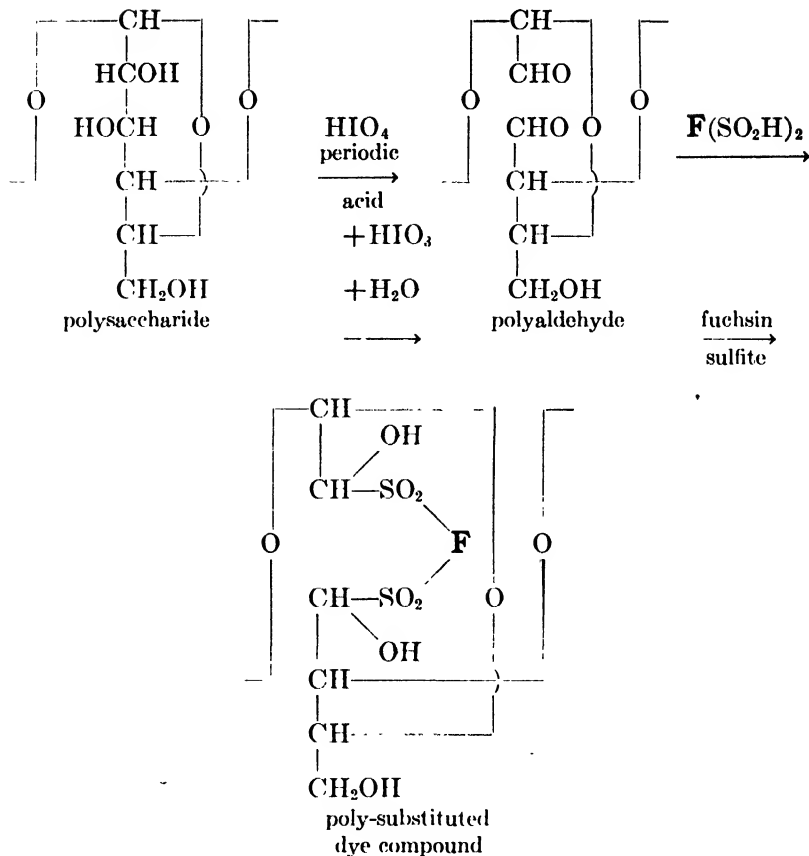
## INTRODUCTION

A consideration of the methods available for the histological identification of carbohydrates suggested that an advantageous new method might be devised based upon the unique properties of the oxidant, periodic acid. This reagent is known to attack polysaccharides, converting them to high-molecular aldehyde compounds (1). It has proved quite possible to oxidize the polysaccharide of tissue preparations to a polyaldehyde which stains fairly intensely with fuchsin-sulfurous acid (Schiff's reagent as used in Feulgen staining). In the somewhat limited experience of the author, the procedure described herewith yields excellent results with various plant materials and results that appear to be very promising with animal tissue preparations. The method outlined here has already been successfully employed in histochemical studies (2, 3).

### *Principle of the Reaction*

The mechanism of the reaction may be illustrated by the reaction of an anhydroglucose residue of starch as shown on page 132. The oxidation is effected very rapidly (in less than 5 minutes, normally) at weakly acid pH, at room temperatures. Although periodic acid is an oxidant potent enough to break the carbon-carbon bond within the monosaccharide residues, it has, remarkably, a negligible tendency to oxidize aldehyde groups. As will be seen later, the possibility of the

<sup>1</sup> The work reported here was largely carried out from January to June, 1945, while the author was serving in the Hospital Corps, U. S. Naval Reserve. The Navy Department does not necessarily endorse the views expressed here.



reaction occurring with a given polysaccharide can be predicted rather precisely from a knowledge of its chemical structure.

While this manuscript was being prepared, a note appeared by McManus on the histological identification of mucin with periodic acid (4). His article gives brief directions for a staining method so similar to the one presented here that it will certainly result in the staining, not only of mucin, but of other polysaccharide-containing elements as well. The present paper is offered since it contains a more extended discussion of the specificity of the periodate-fuchsin staining in relation to chemical structure, together with more complete directions for a procedure that allows staining of certain soluble polysaccharides as well. In addition, certain interferences are removed, and controls provided, in the method outlined.

A similar principle must be involved empirically in the polysaccharide staining method of Bauer (5) which depends upon prolonged

oxidation, under strongly acid conditions, by chromic acid followed by treatment with Schiff's reagent. However, relatively little is known of the mechanism of chromic acid oxidation, and it is not possible to predict in any detail what substances will give the reaction. It appears that the aldehyde groups produced by chromic acid are, to varying degrees, destroyed by further oxidation if incubation is too prolonged. In these respects, periodic acid would seem to offer an improved means of obtaining the same type of staining, since its action, even at very moderate temperatures and acidities, is rapid, clear-cut, and predictable.

### *Specificity of the Reaction*

A positive result in the periodate-fuchsin staining procedure is given by any substance which satisfies all of the following requirements:

- (a) contains the "1,2-glycol" grouping  $\text{—CHOH—CHOH—}$  in unsubstituted form (or the equivalent structure in which hydroxyl groups are replaced by amino or alkylamino groups) or its oxidation product  $\text{—CHOH—CO—}$ ;
- (b) does not diffuse away in the course of tissue fixation;
- (c) gives an oxidation product which is not diffusible; and
- (d) is present in sufficient concentration originally to give a detectable final color.

All of these factors contribute to the specificity of the staining method. The carbohydrate component of ribo- and desoxyribonucleic acids, and the hydroxyamino acid residues of proteins (probably excepting hydroxylysine) are chemically substituted so that the free glycol grouping is not present, and do not give the reaction. Indeed, while many synthetic organic compounds would satisfy requirement (a) virtually all of the known naturally occurring substances which do so (simple and polysaccharides, glycoproteins, mucoproteins, phosphorylated sugars, inositol derivatives, cerebrosides) are classed with the carbohydrates. Of these only the high-molecular substances are left in tissue preparations after ordinary fixation, although it is probable that cerebrosides and inositol-containing lipids are not completely removed by the aqueous fixatives. In the staining procedure the choice of the 70% alcoholic periodic acid described would probably complete the removal of these lipid substances. Furthermore, it appears that only the high-molecular substances (polysaccharides,



hyaluronic acid, mucoproteins, mucins) are likely to be present in adequate amounts to give visible color. The amount of dye fixed is dependent upon the actual weight of glycol structure present, and these substances carry it in greater concentration, and in more insoluble form, than other recognized constituents.

Solutions of isolated substances may be empirically tested for the capacity to give the periodate-fuchsin staining by using the actual staining solutions as "spot-test" reagents. Table I gives the results of tests performed upon a number of purified materials in this way.

TABLE I

*Preparations Reacting with Periodate-Fuchsin in "Spot Tests"*

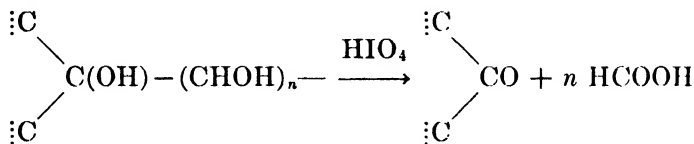
<i>Vigorous reaction with</i>	<i>Weak reaction with</i>
Muscle glycogen	Cellulose
Liver glycogen	Crystalline serum albumin
Hyaluronic acid	Crystalline egg albumin
Gastric mucin	Glucose
Umbilical cord polysaccharide	Glucosamine
Chitin	Glucose-1-phosphate
Crude serum albumin	Galactose
Crude casein	Maltose
<i>Pneumococcus</i> Type III polysaccharide	Sucrose
Friedlander Type B polysaccharide	Xanthosine
Algin	Adenosine
Lemon pectin	Muscle adenylic acid
Gum arabic	Phlorizin
Gum tragacanth	
Glycerol	
Serine	<i>Negative reaction with</i>
Dihydroxyacetone	
Ribose	Washed agar
Arabinose	Ribonucleic acid
$\alpha$ -Glycerophosphate	Desoxyribonucleic acid
Mannitol	Cellobiose
Tartaric acid	Inositol
Gluconic acid	Malic acid
	Tyrosidine
<i>Moderate reaction with</i>	<i>Periodic acid destroyed by</i>
Starch	Tryptophan (brown color)
Glucuronic acid	Glutathione
<i>Pneumococcus</i> Type I polysaccharide	Ascorbic acid
<i>Pneumococcus</i> Type II polysaccharide	Catechol

*Exceptions and Interferences*

If a tissue preparation is found to be stained by fuchsin-sulfite without previous periodic acid oxidation, the alcohol extraction outlined by Feulgen and Voit (6) for removing "plasma logen" may be employed. The usual pentoses and hexoses do not contain an aldehyde group reactive with Schiff's reagent before oxidation.

Iodates or periodates will give red coloration with Schiff's reagent. It is believed that periodate, or iodate formed from it, may be carried over from the oxidizing solution, particularly by combining with metals such as calcium or potassium which may be present in the tissue preparation, and thereby give rise to a serious interference. An acid reducing rinse is provided which effectively removes this possibility.

Among the low-molecular compounds, polyalcohols, such as the sugars or inositol derivatives (if present after fixation), are oxidized by periodic acid to give two aldehyde groups corresponding to the first and last  $\text{—CHOH—}$  groups of a chain (7). Unsubstituted inositol is somewhat exceptional in having no "terminal"  $\text{—CHOH—}$  groups, and each carbon atom is converted into formic acid. In the case of a chain of hydroxyl-substituted carbon atoms ending with a tertiary hydroxyl, this end would give rise to a ketone, which does not readily react with Schiff's reagent:



A consideration somewhat more important for histological or cytochemical work is the fact that a few polysaccharides react with difficulty or not at all with periodic acid. A few polypyranosides, for example, agar, are known, which contain glycoside linkages on carbon atom number three of a large proportion of the sugar residues (8), so that these residues lack the oxidizable glycol grouping. In the specific capsular polysaccharide of Type III *Pneumococcus*, the glucuronic acid residues constituting one-half the molecule are linked in this fashion (9). Two polysaccharides, a polyglucose from yeast (10), and laminarin, are believed to contain only residues linked through the third carbon atom. Laminarin was found to react slightly with periodic acid, however, presumably at the terminal sugar residues (11). Since

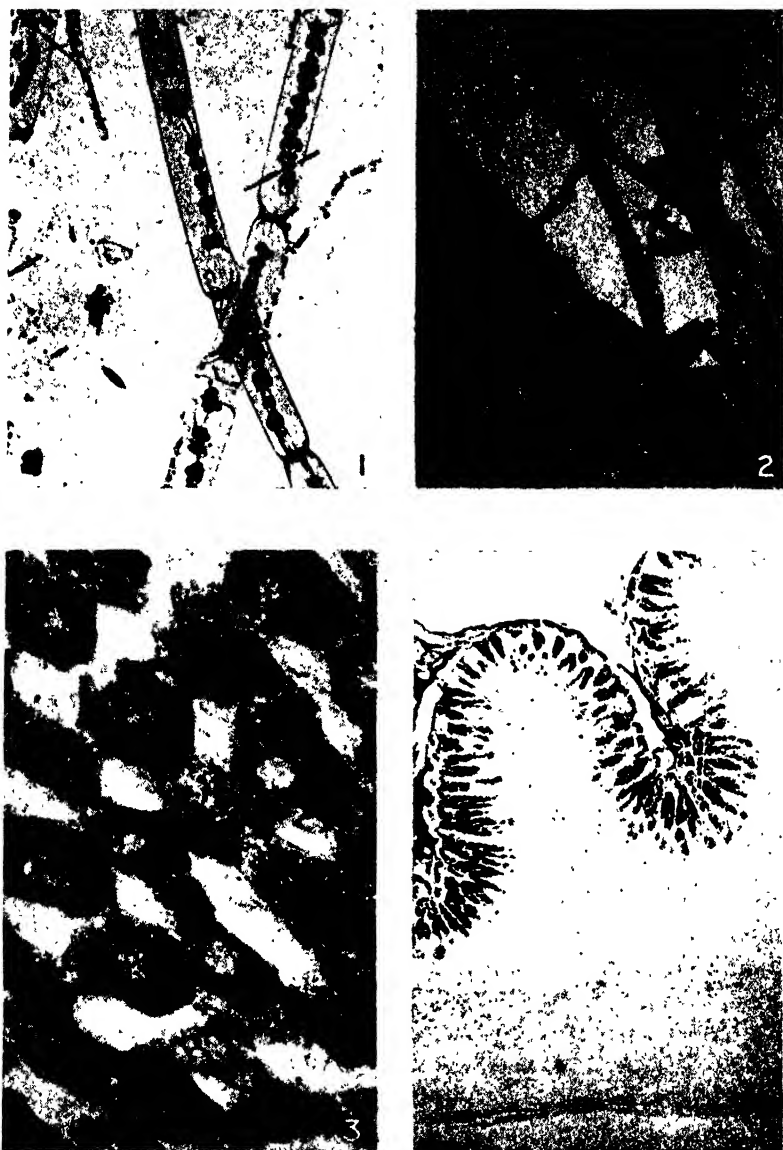


FIG. 1

FIGS. 1 and 2. All tissues shown were stained with periodate-fuchsin only. (Photographs by J. B. Haulenbeek.) 1. Green algae 160X. 2. Mold 750X. 3. Rat liver

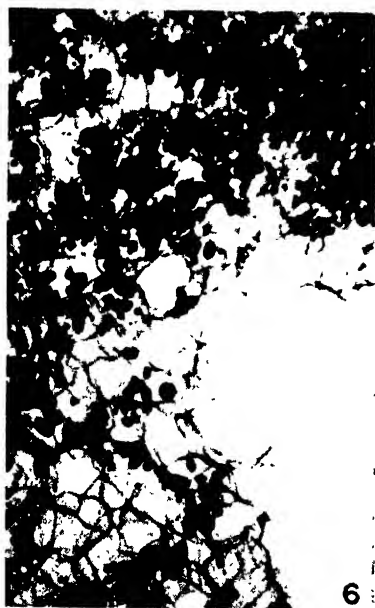


FIG. 2

750 $\times$ . 4. Gastric mucosa 30 $\times$ . 5. Protozoan 750 $\times$ . 6. Potato (freshly cut) 70 $\times$ .  
7. Yeast cells 750 $\times$ .

other polysaccharides of similar resistance may exist, the failure of staining may not safely be taken as demonstrating beyond doubt the absence of appreciable amounts of polysaccharide. Whenever possible, purified or isolated substances should be examined in a "spot test" to suggest optimal conditions for the oxidation of these particular substances.

The reaction was first studied in the hope that it could be applied for the visualization of polysaccharides in bacteria. Although sedimented bacteria acquire a brilliant color when stained in the centrifuge tube,<sup>2</sup> the amount of dye bound by a single cell is, in our hands, only sufficient to make the cell or capsule show up very faintly under the microscope. Yeast cells and fungi are, however, clearly stained, and the "ghost" envelope of a damaged yeast cell can be readily seen.

For usual plant and animal tissue preparations, the staining with fuchsin after periodic acid appears to have useful possibilities. Typical results are shown in Figs. 1 and 2. Plant materials are brilliantly stained, in general revealing cellulose or cellulose-like walls of the individual cells and stored carbohydrate such as starch granules, especially in the region of the chloroplasts if these are present. The cell walls of the freshly cut potato reveal every fold, wrinkle or tear.

With animal preparations, accumulations of polysaccharides are less common. Mucin (because of its polysaccharide content) is strongly stained, as already reported by McManus (4). An objective of the present work was to obtain staining by solutions that would not remove the rather soluble animal polysaccharide glycogen. This is believed to be possible in the buffered alcoholic solutions described below. A photograph of a section of rat liver showing glycogen stained by periodate-fuchsin is found in Fig. 1. In this case, as in all the other illustrations, all the elements that can be seen, except some refractile bodies accompanying the green algae, are visible by virtue of the staining described here.

## EXPERIMENTAL

### *Procedure*

Fixation may be in usual fixatives; mercury salts, if used, are removed with iodine; formaldehyde, if used, is removed by thorough washing. If glycogen, or other easily

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<sup>2</sup> Goebel has already made use of the observations reported here to demonstrate the oxidation of polysaccharide in *Shigella paradysenteriae* and fractions derived from it (12).

soluble polysaccharide, is to be demonstrated, fixation and washing should be in alcoholic or other fluids that do not dissolve this substance. Approximately 70% alcohol has successfully been used for washing in such cases, and the standard procedure suggested here calls for this environment up to the stage at which fuchsin-sulfite is used. Whenever such precautions are unnecessary, aqueous solutions may be used.

After bringing the section or smear into alcohol:

- (a) leave 5 minutes at room temperature in periodic acid solution A;
- (b) flood with 70% alcohol, transfer to reducing rinse, leave 5 minutes;
- (c) flood with 70% alcohol, leave 15–45 minutes in fuchsin-sulfite;
- (d) wash 2–3 times with  $\text{SO}_2$ -water as for Feulgen staining, dehydrate and mount as usual;
- (e) counterstaining (if desired): If staining is mainly to show polysaccharides, counterstain with a basic dye. Malachite green in dilute aqueous solution (about 2 mg./100 cc.) has been satisfactory for some preparations. This will tend to stain the nucleic acids, which are not affected by the periodate-fuchsin. If staining is intended for mucin or acid polysaccharides, it is presumably better to counterstain with an acid dye; and
- (f) control sections are carried through the same process, eliminating step (a).

### *Solutions Used*

*Periodic Acid A.* Four hundred mg. periodic acid, dissolved in 10 cc. distilled water. add 5 cc. of *M/5* sodium acetate (equivalent to 135 mg. of the hydrated crystalline salt) and 35 cc. ethyl alcohol. (The periodic acid,  $\text{H}_5\text{IO}_6$ , was purchased from the G. Frederick Smith Chemical Co., Columbus, Ohio.) This solution may be used for several days if protected from undue exposure to light.

*Periodic Acid B.* Four hundred mg. periodic acid dissolved in 45 cc. distilled water, add 5 cc. of *M/5* sodium acetate.

*Reducing Rinse.* One g. potassium iodide, 1 g. sodium thiosulfate pentahydrate are dissolved in 20 cc. distilled water. Add, with stirring, 30 cc. ethyl alcohol, and then 0.5 cc. 2 *N* hydrochloric acid. A precipitate of sulfur slowly forms and is allowed to settle out, although the solution may be used immediately. (This is designed to be an iodide-thiosulfate solution containing the maximum amount of mineral acid compatible with the thiosulfate; when it ceases to be acidic, it should be re-acidified or replaced.)

*Fuchsin-Sulfite.* This may be as used for the Feulgen stain. The solution prepared as follows is satisfactory. Two g. basic fuchsin are dissolved in 400 cc. boiling water, cooled to 50°C. and filtered. To the filtrate are added 10 cc. 2 *N* hydrochloric acid and 4 g. of potassium metabisulfite. Stopper and leave in the dark in a cool place overnight. Add 1 g. decolorizing charcoal, mix and filter promptly. Add up to 10 cc. or more 2 *N* hydrochloric acid in small portions until, after the last addition, the mixture, spontaneously drying in a thin film upon a glass slide, does not become pink. Preserve in the dark, well stoppered.

*Sulfite Wash Water.* Fifty cc. distilled water containing 0.5 cc. concentrated hydrochloric acid and 0.2 g. potassium metabisulfite.

*Modifications.* As mentioned, aqueous solutions may be used, whenever insoluble polysaccharides only are of interest. Aqueous periodic acid (solution B) is usually somewhat more rapid and vigorous in its action than solution A.

Rinsing is needed to remove entrapped or combined periodate or iodate; either salt gives a reddish coloration with fuchsin-sulfite. While a somewhat longer rinse in 70% alcohol (or water) may frequently be adequate to remove these salts, the reducing rinse is more positive in its action. It would probably be dangerous to use acid iodide and thiosulfate in separate solutions, since the liberated iodine might destroy aldehyde groups if not immediately reduced by thiosulfate. Such substances as glucose or ethylene glycol reduce periodate but not iodate.

If control sections reveal that free tissue aldehydes are present before oxidation with periodic acid, treat tissues as directed by Feulgen and Voit (6).

Excessive egg white used in coating slides, if not well drained, contains enough carbohydrate to accept a barely perceptible stain.

Preparations stained and mounted when this work was begun are still apparently unchanged 2.5 years later.

### *Spot Tests with Periodate-Fuchsin*

Solutions containing approximately 1 mg. of various preparations are placed in a spot plate and treated for 5 minutes with one drop of periodic acid. Toward these aqueous solutions, the fresh alcoholic solution A is fully as vigorous as solution B and the drops are of a more convenient size. Because the periodic acid (and acetate buffer) is not removed, an appropriate small excess of dilute hydrochloric acid is added immediately before the reducing rinse. A periodic acid blank reduced in this way should give no coloration with Schiff's reagent, and also not interfere with color development when a drop of very dilute formaldehyde is added.

Satisfactory proportions are:

- (a) water or polysaccharide solution, 0.05–0.2 cc.
- (b) periodic acid solution A, 0.025 cc. (or 1 drop). Leave 5 minutes.
- (c) N/10 hydrochloric acid, 0.05 cc. (or 1 drop).
- (d) reducing rinse (alcoholic), 0.1 cc (or 4 drops). Mix.
- (e) fuchsin-sulfite reagent, 0.05 cc. (or 1 drop). Leave 15 minutes.

Similar proportions of the aqueous solutions may be used, making allowance for larger drop size.

Sluggish color development, resulting in fainter colors, may be due to slower reaction with fuchsin-sulfite found with some aldehydes, and is not usually improved by prolonging the periodic acid oxidation. Nevertheless, with substances giving a negative reaction, consideration should be given to modifying the conditions for the oxidation. For substances giving a positive reaction it is necessary to consider the possibility that the effect is given by impurities present.

Most of the substances recorded as reacting in Table I give marked color after standing 1–2 minutes with 0.05 cc. of fuchsin-sulfite.

Adding more of the Schiff's reagent will tend to overcome the effect of too high acidity.

#### ACKNOWLEDGMENT

It is a pleasure to acknowledge the assistance of Katharine Migel Ayerigg in this investigation. Mrs. James K. Alexander and Myrma Spoerl very kindly provided a number of tissue sections for which I am greatly indebted to them.

#### SUMMARY

Polysaccharides are oxidized under mild conditions by periodic acid to give polyaldehydes which yield colored compounds with Schiff's reagent, fuchsin-sulfite. In solutions this reaction can be obtained with a wide variety of natural polysaccharides. With suitable precautions it may be used for the semipermanent staining of starch, cellulose, glycogen, mucins, chitin, *etc.*, in plant and animal tissue preparations. The chemical reactions leading to the staining are rather completely known, and the outcome is, in general, predictable, if there is knowledge of the chemical structure.

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# The Amino Acid Metabolism of *Penicillium chrysogenum* Q-176

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Received August 8, 1947

## INTRODUCTION

Nutritional aspects of the growth of *Penicillium notatum* and *P. chrysogenum*, and those dealing more particularly with the production of penicillin by these organisms, have been studied by numerous investigators. Early studies of penicillin (4, 12, 1) employed a modified Czapek-Dox solution, with  $\text{NaNO}_3$  as the source of nitrogen. Foster *et al.* (9) were apparently the first to attempt to use individual amino acids as sole sources of nitrogen in the production of penicillin by *P. notatum*, concluding that, in their experiments with surface cultures, nitrate was superior to amino acids.

With the introduction of corn steep liquor as an ingredient of media for penicillin production (5), large increases in the yield of penicillin were obtained. White, Krampitz and Werkman (15) found that the stimulatory activity of corn steep liquor was largely due to arginine, histidine and glutamic acid. When these compounds, in concentrations of 30 mg., 30 mg., and 400 mg. per 100 ml., respectively, were added to a basal medium containing glucose, lactose and mineral salts, yields assaying 80–90% of that obtained on corn steep media resulted. This suggested the possibility that amino acids are involved in the biosynthesis of penicillin: "It would appear that in some way this mold is perhaps able to convert one or several of the amino acids to a part of the penicillin molecule through its metabolic processes" (15).

Bonner (3) tested alanine, serine, valine, cystine, and methionine as possible penicillin precursors in surface and submerged cultures. Experiments using mutant penicillinless strains, to which these compounds were supplied singly, in the presence of phenylacetic acid, and in the presence of *dl*-penicillamine, yielded completely negative results.

Stone and Farrell (14) failed to obtain consistent increases in penicillin yield upon the addition of most of the common amino acids to synthetic media, although occasional increases sometimes followed the addition of leucine, cystine or cysteine. Cook and Brown (6) recorded increased yields upon the simultaneous addition of glucose and leucine or glucose and tyrosine, although the addition of the amino acids

alone gave only a slight stimulatory effect. Halpern *et al.* (10) reported marked stimulation of growth and increased penicillin production upon the addition of proline or glutamic acid to synthetic media, but a number of other amino acids, including histidine, methionine, tryptophan, hydroxyproline, and phenylalanine, were ineffective. It has recently been reported (8) that yields in submerged cultures can be raised by the addition of phenylacetyl derivatives of various amino acids.

In contrast to these studies of the effects of amino acids upon the yield of penicillin, relatively little is known concerning the role of amino acids in the growth and other metabolic activities of the fungi concerned. That penicillin formation is not necessarily closely correlated with growth of *P. notatum* and *P. chrysogenum* is shown by observations that on certain media these fungi make excellent growth, yet produce negligible amounts of penicillin.

Dimond and Peltier (7) observed that *P. notatum* grew more rapidly on a medium containing asparagine, tryptophan and cystine than on a medium containing nitrate, and suggested that, in media containing both amino and nitrate nitrogen, a preferential use of the amino acids occurred. Stokes and Gunness (13) have presented analyses for the "essential" amino acids obtained by microbiological assay upon hydrolyzates of mycelia of *P. notatum* grown under a variety of conditions. The results disclose a high value for leucine, low contents of methionine and tryptophan, and do not differ appreciably from findings obtained with a number of other microorganisms.

The present study was undertaken to supplement previous findings concerned with carbohydrate metabolism in a surface strain of *P. notatum* (16) and to provide information concerning the amino acid metabolism of *P. chrysogenum*. The oxidation of various amino acids by *P. chrysogenum* was studied by means of respirometer techniques.

## MATERIALS AND METHODS

*Penicillium chrysogenum* Q-176 was the organism employed in the present experiments. It was originally produced by ultraviolet irradiation of strain X-1612 (Backus, Stauffer and Johnson, 2). Strain Q-176 is capable of producing 900 Oxford units of penicillin/ml. under suitable conditions (2), and is the strain employed almost exclusively in commercial production of penicillin at the present time.

Stock cultures were maintained upon the "Sporulation Medium" of Moyer and Coghill (11a), solidified by the addition of 2.5% agar. The medium used in submerged cultures, also developed by Moyer and Coghill (11b), has the following composition: corn steep liquor (55% solids), 40 ml.; lactose monohydrate, 27.5 g.; NaNO<sub>3</sub>, 3.0 g.; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.25 g.; KH<sub>2</sub>PO<sub>4</sub>, 0.50 g.; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.044 g.; MnSO<sub>4</sub>·4H<sub>2</sub>O, 0.020 g.; and glucose monohydrate, 3.0 g. per liter. Cultures were grown at room temperature in Erlenmeyer flasks of 500 ml. capacity containing 125 ml. of medium. The cultures were continuously agitated by a reciprocating shaker which made 100 strokes per minute with a stroke length of 7.0 cm.

Measurements of respiration were made with the use of a Warburg respirometer of conventional design. Experiments were conducted at a constant temperature of

27.0°C., which closely approximates the optimum for growth and penicillin production by this organism. Following a large number of preliminary trials, 0.15 *M* Sørensen's phosphate buffer having a pH of 6.9 was selected for use in all experiments.

The respiration experiments were performed using cultures that had been grown on the corn steep medium for 6½ days. At this time, the submerged mycelial pellets were removed from the culture solution by filtration, washed in running water for a period of 30–150 minutes to deplete the food reserves, and suspended in buffer.

Two-tenths ml. 10% KOH within the center wells of each respirometer vessel provided for the absorption of carbon dioxide. In each experiment, four vessels were designated as experimental, containing fungus mycelia and a *M*/100 solution of the particular amino acid to be tested (dissolved in 0.15 *M* Sørensen's phosphate buffer). Two vessels were employed as controls for the measurement of the endogenous respiration of the fungus in buffer without added substrate, the remaining vessel serving as the thermobarometric control. Following equilibration of temperature and pressure, measurements of the oxygen consumption were made at 10-minute intervals for one hour.

At the completion of each experiment, the mycelia from each vessel were decanted into tared filter papers, were rinsed with distilled water to remove the remaining traces of substrate and buffer, and the filter papers containing mycelia were dried to constant weight in a desiccator over drierite. The  $Q_{O_2}$  was computed for each of the four experimental and two control vessels in each experiment.

## RESULTS

Results were obtained upon 23 amino acids, all of which were supplied in a concentration of *M*/100. Data from 55 experiments of the type just described are assembled in Table I. In evaluating the effects of the various amino acids upon the respiration of *P. chrysogenum*, the average  $Q_{O_2}$  (mm.<sup>3</sup> O<sub>2</sub>/hr./mg. dry weight) of the two control vessels without substrate was compared with the average  $Q_{O_2}$  of the four vessels containing *M*/100 amino acid. To facilitate comparison of different substrates, the results are also expressed as percentage increase in oxygen consumption due to the presence of the particular substrate in question.

## DISCUSSION

As is apparent from Table I, considerable variation among the different experiments was found in the values obtained for the original  $Q_{O_2}$ , as measured in the absence of substrate. These variations are believed to be primarily due to the fact that the cultures were not grown under conditions of constant temperature. Variations in the time allowed for washing the mycelia in water to deplete the food reserves was found to have no significant effect upon the  $Q_{O_2}$ .

TABLE I  
*The Action of Amino Acids on the Oxygen Consumption of  
 Penicillium chrysogenum*

Amino acid (M/100)	Original $Q_{O_2}$	Final $Q_{O_2}$	Percentage increase in oxygen consumption
L(-)-Cystine <sup>1</sup>	8.89	8.28	0
	4.86	4.41	0
	6.50	5.51	0
Glycine (aminoacetic acid)	5.14	5.40	5
	4.20	4.40	5
DL-Valine	5.17	5.43	5
	4.71	5.05	7
DL-Isoleucine	7.73	7.90	2
	4.52	5.68	26
	4.93	5.09	3
L(+)-Aspartic acid	8.53	9.25	8
	5.10	5.45	7
	3.91	4.12	5
L(+)-Cysteine · HCl <sup>2</sup>	7.42	8.30	12
	8.97	9.63	7
	3.93	4.19	7
L(+)-Lysine · HCl	5.96	6.62	11
	7.80	9.26	19
L(+)-Histidine · HCl	4.22	4.98	18
	6.29	7.36	17
L(-)-Asparagine	5.38	6.59	22
	6.00	7.35	23
L(-)-Leucine	5.04	5.92	17
	4.43	5.49	24
$\beta$ -Alanine	4.82	5.61	16
	4.67	5.82	25

<sup>1</sup> Incompletely soluble in M/100 solution.

<sup>2</sup> The data presented represent differences between experimental determinations and controls containing cysteine · HCl but no fungus mycelia, to eliminate oxidation of the substrate by atmospheric oxygen.

TABLE I (Continued)

Amino acid (M/100)	Original $Q_{O_2}$	Final $Q_{O_2}$	Percentage increase in oxygen consumption
L(-)-Tyrosine <sup>1</sup>	5.44	7.01	29
	5.53	6.83	23
DL-Phenylalanine	6.21	8.29	33
	5.67	6.76	19
	6.87	8.83	29
DL-Norleucine	6.37	8.85	39
	6.01	7.38	23
L(-)-Tryptophan	6.32	8.20	30
	5.04	6.50	29
L(+)-Arginine · HCl	5.03	6.88	37
	6.47	8.24	28
DL-Serine	5.40	6.70	24
	3.11	4.34	40
L(-)-Hydroxyproline	4.17	5.48	31
	3.78	5.07	34
DL-Threonine	4.68	6.44	38
	6.07	8.24	36
DL-Methionine	5.45	7.26	33
	8.85	13.87	57
	7.17	9.28	29
L(-)-Proline	4.28	6.92	62
	5.12	7.38	44
	4.00	6.16	54
L(+)-Glutamic acid	3.63	6.61	82
	6.23	8.00	28
	3.34	6.35	90
DL-Alanine	5.62	10.89	94
	3.34	7.31	119
	6.17	10.54	71

It is noteworthy that this strain of *P. chrysogenum* is able to make use of a large number of amino acids. As might be anticipated, individual amino acids vary greatly in their abilities to increase the oxygen consumption, and presumably the growth, of this fungus. Cystine does not appear to be oxidized by *P. chrysogenum* under the experimental conditions employed. Glycine, valine, isoleucine, aspartic acid and cysteine are very slowly oxidized by *P. chrysogenum*, resulting in increases of 10% or less in the rate of oxygen uptake.

A large number of amino acids, including lysine, histidine, asparagine, leucine,  $\beta$ -alanine, tyrosine, phenylalanine, norleucine, tryptophan, arginine, serine, hydroxyproline, threonine, and methionine, are oxidized fairly rapidly, giving rise to increases of approximately 15–40% in the oxygen consumption of *P. chrysogenum*. Proline, glutamic acid, and alanine consistently gave large increases in the oxygen consumption of *P. chrysogenum* and are oxidized very rapidly by this organism.

When the present results are compared with the data of others concerning the stimulation of penicillin yield by various amino acids, close agreement with the findings of Halpern *et al.* (10) is to be noted. Proline and glutamic acid were found by these workers to cause increased penicillin production by *P. notatum* 832 when added to synthetic media, and they are said to be the amino acids present in highest concentration in corn proteins. Proline and glutamic acid, in our experience, are among the amino acids most rapidly oxidized by *P. chrysogenum* Q-176. These compounds would, therefore, appear to be of considerable significance in the nutrition of penicillin-producing organisms.

It is to be hoped that a further knowledge of amino acid metabolism may eventually result in an understanding of the mechanisms involved in the biosynthesis of penicillin.

#### ACKNOWLEDGMENTS

This work was made possible by grants from the Carnegie Foundation for apparatus and chemicals, and from the Natural Science Research Fund of Vanderbilt University for technical assistance. Miss Jamie Harris, Mr. John O. Batson and Mr. Richard C. Smith have assisted with the experiments. The author also wishes to express his appreciation to Dr. Kenneth B. Raper of the Northern Regional Research Laboratory for the culture of *P. chrysogenum*, and to Mr. H. A. Kaufmann of the American Maize Products Company for the corn steep solids used.

## SUMMARY

The oxidation of 23 amino acids by *Penicillium chrysogenum*, strain Q-176, was studied by means of oxygen consumption measurements made with the Warburg respirometer. Cystine is not oxidized by *P. chrysogenum* under the conditions of these experiments. Glycine, valine, isoleucine, aspartic acid, and cysteine are very slowly oxidized by *P. chrysogenum*. Lysine, histidine, asparagine, leucine,  $\beta$ -alanine, tyrosine, phenylalanine, norleucine, tryptophan, arginine, serine, hydroxyproline, threonine, and methionine give rise to moderate increases in the rate of oxygen uptake. Alanine, glutamic acid, and proline are rapidly oxidized by *P. chrysogenum*.

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## LETTER TO THE EDITORS

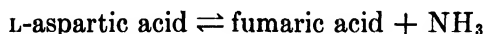
### Estimation of L-Aspartic Acid with Aspartase

Sirs:

As the first enzymatic method for the quantitative determination of amino acids, Virtanen and Laine (1) applied aspartase for determination of L-aspartic acid. The method was then used as a macro method and its accuracy was not tested in detail. We have now developed the method for estimation of small amounts of aspartic acid and have investigated in detail the factors affecting the result.

The experiments were made both with finely ground preparation of dried *Pseudomonas fluorescens*, and with the enzyme solution extracted from the bacterium. The use of the dry preparation is most rapid and convenient. The preparation maintains its full activity for half a year at least, probably for years. Phosphate buffer, pH 7.0, was employed in the experiments.

Because the bacterium preparations contain also fumarase which affects the equilibrium



the following 3 parallels are necessary for carrying out the determinations.

1. Experiment with bacterium preparation without aspartic acid to enable determination of  $\text{NH}_3\text{-N}$  set free from the preparation.
2. Experiment with bacterium preparation with a known amount of L-aspartic acid for determination of  $\text{NH}_3\text{-N}$  liberated from aspartic acid during the experimental period (*e.g.*, 0.5 mg. N).
3. Experiment with bacterium preparation with the solution to be investigated, the aspartic acid content of which must correspond as nearly as possible to that in case 2 for obtaining of accurate results.

The experimental period must be exactly the same in each parallel experiment. The following example illustrates our method (Table I).

TABLE I

*Determination of Aspartic Acid from the Casein Hydrolyzate (Casein Schering Kahlbaum acc. to Hammarsten) by Dry Preparation of Ps. fluorescens. Period of Experiment 2 hr., Temperature 34°C.*

No. of expt.	L-Aspartic acid-N	N in casein hydrolyzate	NH <sub>2</sub> -N formed	NH <sub>2</sub> -N split off from aspartic acid		L-Aspartic acid-N in casein	
							Per cent total N
	mg.	mg.	mg.	mg.	Per cent	mg.	
1	—	—	0.427	—	—	—	—
2	—	—	0.433	—	—	—	—
3	—	—	0.441	—	—	—	—
			Av. 0.434				
4	0.538	—	0.851	0.417	77.51	—	—
5	0.538	—	0.840	0.406	75.46	—	—
6	0.538	—	0.843	0.409	76.02	—	—
			Av. 0.845	0.411	76.39		
7	—	11.85	0.965	0.531	(76.39)	0.695	5.86
8	—	11.85	0.972	0.538	(76.39)	0.704	5.94
9	—	11.85	0.951	0.517	(76.39)	0.677	5.71
			Av. 0.963	0.529	76.39	0.692	5.84

A detailed description of the method will be published in *Acta Chem. Scand.*

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## Book Reviews

**Radioactive Tracers in Biology.** By MARTIN D. KAMEN. Academic Press, New York, 1947. ix + 281 pp. Price \$5.80.

It is fortunate, indeed, that the first book to make information about radioactive tracers available to biologists should be written by Professor Martin Kamen. For Professor Kamen's work in this, his field, is most outstanding in both the physical and the biological sense. With Samuel Ruben he was responsible for the discovery of  $C^{14}$ , the long-lived radioactive carbon which will probably be the most useful isotope of them all for the biologist. Furthermore, with Ruben and Hassid, he first used  $C^{11}$  to prove directly that  $CO_2$  is fixed by plants.

Out of some 250 pages, the first 50 are devoted to a general discussion of radioactivity; the next 75 treat of the techniques for measurement, and a general discussion of the possibilities of "tracer methodology." The remainder of the book covers in great detail the isotopes most useful for biological work, with examples selected from Kamen's own work and the literature in general. The last chapter, a scant 13 pages, covers medical applications and radioautographs.

Some small understanding of the physical processes involved in radioactivity is an essential for the biologist who is planning to make use of isotopes for research. The subject is still too young, the techniques still too undeveloped, for any other approach. Traps for the wary and initiate still exist; all the more reason for the inexperienced to tread with caution. The requisite concise and logical introduction to an understanding of these physical processes can be found in the first part of the book.

Kamen has chosen 21 elements to discuss in detail. Very possibly the most valuable portion of the book is the introduction to each of these sections, in which he discusses each element in detail, setting forth the advantages and disadvantages in the use of each isotope, the methods of manufacture and the necessary techniques for separation, purification, and detection. This information does not exist elsewhere; indeed, in some cases the information has never been published. As a consequence, now for the first time a biologist who wishes to enter the field may learn at once whether the experiment which seems so nice in imagination is at all feasible, without first conducting a long research through periodicals in the separate fields of physics, chemistry, and biology. In other words, a primer for the uninitiate.

The examples of tracer research quoted in the second half of the book are not so unique a contribution, since, as Kamen points out, review articles exist that cover much of this work. The only connection between many of the examples chosen in the book is that they are in the general kingdom of biology, and all make use of the same tool. In the early stages before the tool is fully assimilated into the daily practice of the research scientist, its description cannot be separated from the details of its use. Hence, in a book like Kamen's, the author can never be free of the philosophically unpleasant task of writing about subjects whose unity derives only from use of the same technique.

The summary treatment of the hazards of radioactivity which are covered in only a page and a half does not place sufficient emphasis on the real hazard which may be associated with work with radioactive tracers. Before the war, when the amounts of isotopes available were small, proper health precautions did not seem so necessary. Now that \$58 can purchase a third of a curie of  $P^{32}$ , the importance of proper radiation protection cannot be neglected.

It is always dangerous to emphasize the limitations of growing techniques, as Kamen has done with radioautographs. The work of Leblond has already suggested a method to obtain improved definition in radioautographs—improvement that may well be extended when Leblond's and related techniques are applied to  $C^{14}$  with its soft radiation.

There is one important matter of technique in which the reviewer differs markedly from Kamen. The method for quantitative assay of  $C^{14}$  proposed is so cumbersome that it may serve to disenchant biologists with the possibilities of  $C^{14}$  research. Fortunately, precise results may now be attained in these assays using methods far less complex. It is unfortunate that Kamen's involved style of exposition seems to increase the difficulties in whatever technique he describes.

Nonetheless, these objections are all minor. The important and outstanding fact is this: Professor Kamen has written an accurate book, a fine book, and a necessary book for any biologist about to embark on the fascinating research now newly possible with the aid of isotopic tracers.

A. K. SOLOMON, Boston, Mass.

**Gonadotrophines et tumeurs testiculaires.** By M. SORBA, ancien assistant, Surgical Hospital, University of Lausanne. Benno Schwabe et Co., Basle, 1946. 80 pp. Price fr. (Suisse) 6.-

Of the important problem concerning the role played by hormones in cancer, Dr. Sorba discusses only one aspect: the gonadotropic substances found in the urine of patients suffering from cancer of the testis.

Dr. Sorba reports 14 personally observed cases of testicular cancer, and compares his findings with those found in the literature.

There are two types of testicular cancer having different histological structure, and associated with different hormonal conditions. "Seminoma" is accompanied by urinary excretion of hypophyseal gonadotropic hormones while "dysembryoma" (teratoma) of the testicle secretes chorionic luteinizing gonadotropins. In addition, dysembryoma is accompanied by histological changes of the pituitary gland. The gland has the aspect of "pregnancy-gland" and contains few gonadotropic factors. On the other hand, seminoma does not provoke histological changes of the pituitary gland, which retains its normal degree of gonadotropic activity.

There is no strict relation between the histological structure of the testis tumor and its hormonal behavior. At least 25% of the tumors did not exhibit the correspondence between structure and gonadotropins described above. Thus, the histological classification of cancer is not always identical with its biochemical classification. This difference must be borne in mind in cancer investigations.

The book includes all necessary data on the technique of investigation employed.

HENRY K. WACHTEL, New York.

**L'acquisition de la science.** By PIERRE VENDRYÈS. Editions Albin Michel, Paris, 1946. 454 pp. Price 260 fr.

## I

The purpose of this book is to trace the order and development of the activities of mind by which scientific work is carried on. The "acquisition" of science is here regarded in the light of the several disciplines of the intellect which together comprise scientific activity. There is no intention to trace the history of the rise of science in the usual sense, but rather, with the aid of the clearest possible historical illustrations, to distinguish the essential components of scientific thinking. Since these different components have been recognized at different stages in the historical rise of modern science, and have emerged in the contexts of different sciences, the author's discussion of them yields incidentally a rich synopsis of the whole movement of modern science.

In a general way Pierre Vendryès' thesis may be taken to be a defense of the actively creative character of scientific thought, "*le rôle créateur de la pensée*" (p. 371), "*son autonomie créatrice*" (p. 322). "*La faculté de créer est innée à la pensée*" (p. 90). The reader might at first think that this stress upon creativity reflects the influence in French philosophy of science of the thought of Bergson; yet no reference to this thinker occurs in the work, suggesting that the historical context for this dominant idea is to be sought elsewhere. The emphasis upon the elements of creative spontaneity in scientific thinking permits M. Vendryès to associate his primary thesis with a secondary one, namely, that modern probability theory furnishes the means of transition from the causal determinacies of the physical sciences to the chance indeterminacies of the life sciences, including the sciences of human conduct. Thus, the present work on "*L'acquisition de la science*" presents a general philosophy of science in the spirit of the author's earlier work, "*Vie et probabilité*."

M. Vendryès names his point of view: "probabilistic humanism" (*humanisme probabiliste*, pp. 383, 445), in contradistinction to a positivistic humanism like that of Auguste Comte (p. 373). This reviewer has gained the impression that the larger aim of the book is to point out a way of "going beyond" positivism without becoming entangled in metaphysics, or perhaps without becoming entangled in metaphysics very much. But such a general comment requires the support of a *précis* of the author's argument.

## II

In nine chapters are set forth the stages of increasing "intellectual autonomy" by which science is acquired.

"*La curiosité que l'homme porte aux choses est la cause de toute science*" (pp. 9, 322). Curiosity (Chap. I) leads to the Acquisition (Chap. II), and Comparison (Chap. III) of facts, resulting in Natural Classifications. Laws of Nature (Chap. IV) give reasons for the classifications. Principles (Chap. V) emerge as presuppositions of the Laws. Hypotheses are created (Chap. VI), and Theories are constructed (Chap. VII) to give comprehensive organization to large bodies of facts and laws. Finally, through the Critique of Fundamental Concepts (Chap. VIII), the final stage of a Philosophical System (Chap. IX) is reached. "*L'idée commune à tous les chapitres précédents est que la Science est une oeuvre humaine, qui exige une intelligence humaine*" (p. 321). In a word, science is one of the humanities (p. 17).

Each chapter contains distinctive observations on familiar themes.

Curiosity, "*le don de s'étonner*" (p. 10), is normally directed outward upon the external world. Its last object is the thinking self with its curiosity, disclosed reflexively by curiosity about curiosity itself. In this light, the study of "the acquisition of science" takes on a certain introspective character, even while dealing with the public accomplishments of the several sciences.

The initial discipline of curiosity is represented by the descriptive phases of science, as, for example, in descriptive anatomy and descriptive chemistry. Every scientific term shall be based on facts, and no term shall be used for which no corresponding facts can be found. "Scientific language ought to comprehend reality and not be a language of convention" (p. 35). The difficulty of this discipline is well represented by a science like mechanics in which the initial data are by no means the immediate data of sense preception (p. 27).

The art of discipline of comparison, seeking the natural classifications of facts (*e.g.*, the inert gases, the vertebrates), requires, even more than does description, a penetration of the immediately given data of sense to find hidden relations and real analogies. But classifications, however natural, do not explain themselves. Their reasons are formulated in terms of natural laws.

In the author's view, a science of natural laws is a "rational science," to be contrasted with a probability science in which chance displaces causality (pp. 118, 128). He compares a natural law, as an intellectual instrument, to a mathematical theorem. (His illustrations: Galileo's law of falling bodies, and a geometrical theorem involving Simpson's Line.) A law and a theorem alike possess demonstrative rigor. In the case of the theorem, the demonstration is to be taken as a preliminary to the intuitive act of consent in which the mind "grasps" the abstract relationships stated in the theorem (p. 90). The author here shares the views of Poincaré on mathematical creativity and inventiveness (p. 99). In the case of the natural law, demonstration requires for its completion the test of experience since the natural law is concerned, not with abstract relations, but with real relations.

This classical, or rational, conception of a science of natural laws leads historically to a doctrine of universal causal determinism (witness the often quoted assertion of Laplace) (p. 127). But a limitation upon natural law now exists in the theory of chance (p. 134). An alternative to prediction based on causal laws is furnished by prediction based on statistical laws, since "*par la sommation de ses répétitions, l'événement tend, aléatoirement, à perdre ses caractères aléatoires*" (p. 133). A rational science cannot be the whole story about nature, especially in biology; it mistakenly attempts "to comprehend with a philosophy of the solid a fluid nature" (p. 136) (*cf.* Bergson!).

Principles complete the rational structure of laws, as postulates complete the mathematical system of theorems. They articulate the basic invariants which our thinking requires as "an assurance" (p. 146). The author's illustrative material is drawn from mechanics in such principles as those of the conservation of energy, of statics. A principle, unlike a law, states an empirical generalization which "goes beyond" the possibility of demonstration (p. 150). The difference appears to be at once one of degree of empirical generality and, in a Kantian sense, of "purity." (This reviewer has difficulty in reconciling the two parts of this assertion.)

The following stage of ascent, the creation of hypotheses, involves a psychological process for which, in the author's view, there is no strict logical method. The drawing

together of principles, laws, classifications into hypothetical unities is a work of inspiration. The mental process leading to such discoveries is compared to incubation, to crystallization. One is reminded of the emphasis upon trained sagacity in the writings of William Whewell and Augustus De Morgan. The author includes an interesting personal account of the achievement of an illuminating hypothesis in his researches on probability and life.

Science comes of age with the achievement of a theory. Certain hypotheses prove to have such a power of unifying other hypotheses into systems of maximum coherence and empirical fulness, that they stand out as master hypotheses, or theories. Under them, other hypotheses become laws. All the partial disciplines thus far enumerated are brought into play (p. 199). The atomic theory is taken as a preeminent example of this stage of intellectual construction.

The transition from science to scientific philosophy is marked by reflections upon the value of the whole scientific enterprise in a natural environment which science finds to be indifferent and devoid of meaning (p. 216). A critique of fundamental concepts is required for the resolving of the paradoxes of fact and meaning. The author is aware of the Kantian critique wherein the meaning of science derives from the legislative activity of the human reason and will. But wishing to make his principle the creativity of thought, rather than the legislative activity of thought (p. 323), he gives a critique of three concepts, space, chance and time, in such a way as to be rid of the apparatus of the Kantian *a priori*.

With regard to space, the position taken is that it is physical fields of force which are the factual basis from which geometrical space is derived by abstraction. Space is thus not a form of the understanding (*sic!*), neither synthetic nor *a priori*, but is derived solely by analysis from the mechanical fact of a field of force (p. 233).

The treatment of chance is more detailed, extending to 30 pages. The concept "chance" refers to the physical fact of independence between events. Classes of events thus negatively related exhibit the properties of chance, or probability. Statistical probabilities may be formulated, given relative frequencies; but these are not the only kind of probability (p. 243). Unprecedented events also have probabilities. In any case, "probability" is here taken in an objective sense, since the physical relation of independence is objective. The physical context of this relation is "the void," carefully distinguished by the author from "space" as an abstraction from a field of force. The distinction seems to this reviewer artificial. The idea appears to be that "space" serves the purposes of a causality science, and "the void" serves the purposes of a probability science (pp. 260-263).

The critique of time is a brief corollary of that of chance. If the concept of time as continuous is based on a causally continuous sequence of events, then a sequence of independent events would seem to impose on time a fragmentary character. "*L'indépendance réelle des faits doit rompre cette continuité du temps*" (p. 247).

The foregoing naturalistic critique of concepts is offered as a substitute for a doctrine of synthetic judgments *a priori*. "*La réalité est le système de référence de toute notre science*" (p. 268). Even the most fundamental concepts of scientific thinking are "acquired"; no one of them is "inherent in the constitution of our minds" (p. 268).

The philosophical system which emerges from this critique is presented as a rectification of the philosophical synthesis of Comte. The Comtian synthesis was too



narrowly bound to physics, and the resulting conception of science as a rational body of laws of nature. "*Le positivisme n'est qu'un cartésianisme dégénéré et le scientisme un positivisme dégénéré*" (p. 300). In view of the modern uses of probability and of the advances in the life sciences, we must regard natural laws, not as the goal of knowledge, but as instruments of our knowing; then we shall be willing to restrict the use of the methods of "rational science" as we discover the utility of probability science and the theory of chance. The Comtian positivists feared that any efforts to gain knowledge "beyond" laws of phenomena would lead into that unscientific refuge called metaphysics. But a modern life science based on probability shows a way "beyond" laws, in the sense of "rational science," which is compatible with naturalism. "*La nature s'explique par la nature et la métaphysique reste inutile. A ce point de vue, il faut être plus positiviste qu'Auguste Comte*" (pp. 312-313).

Concluding discussions extend the author's view on probability to psychology, ethics and theory of knowledge. There is included also a valuable application of the point of view of the book to the problem of pedagogy in the natural sciences.

The key to the psychological discussion is the idea of subjective probability. The British empirical tradition in psychology is to be rejected in favor of the French psychological tradition. The Cartesian intuitive certainty: "*Je doute, donc ma pensée existe*" is to be amended: "*Mes idées sont probable, donc ma pensée est autonome*" (p. 348). In other words, the thinking mind has no doubt that the relation between its thoughts and its objects is one of independence. The reviewer must add that he finds this assurance enviable, in view of modern epistemological controversy. In terms of the idea of subjective probability, the distinction is developed between creative thought and organizing thought, the former exhibiting the characters of chance, the latter operating according to a program of logical rules. The laws of thought formulated by logic have no absolute authority, and are themselves acquisitions. In the aspects of consciousness which are governed by chance it is possible to develop a scientific account of the will and of moral autonomy, thus completing the program of a "probabilistic humanism."

### III

In the above *précis* of the argument of the book, the reviewer has neglected to give evidence of the remarkable wealth of illustrative material throughout the text. Examples from anatomy, chemistry, geometry, mechanics, abound and are set forth with elegance. References to a wide range of classical philosophers and scientists (in particular, Poincaré and de Broglie—to whom the book is inscribed) occur throughout. Their aptness is such that the reader must regret there are no footnotes by which to trace them to their origins, and that there is no index by which to recover them easily. One notices that the quotations document strongly the French intellectual tradition. It is as though, during the period of the war (the manuscript was finished in 1944), there was occurring among French scientists a resurgence of appreciation of insights specifically French. As an example, the author recommends his philosophy of probabilistic humanism as based upon three sciences "eminently French": biology (Réaumur, Buffon, Lamarck, Cuvier, Geoffroy-Saint-Hilaire, Claude Bernard, Pasteur, . . .); introspective psychology (Montaigne, Descartes, Pascal, Bossuet, the moralists of the 17th century, Maine de Biran, . . .) and the theory of probability (Fermat, Pascal, Laplace, Poisson, Cournot, . . .) (p. 383). The wholly under-

standable sense of intellectual patriotism is not such as to exclude a wider sense of the other national traditions of Europe. The New World, however, lies quite beyond the horizon still.

This last point is the more remarkable since the point of view of the author has notable similarities to instrumentalist and pragmatic strains in American thought. The linking of the theory of chance with the problem of freedom recalls James's essays having the same intention (despite the author's distortion of James—p. 374). The instrumentalist aspect of the treatment of laws and hypotheses as intellectual tools, created by thought for the comprehending of nature, is unmistakable. Even the reaction against Kant resembles the critique of the Kantian critique to be found in American pragmatism.

Other particular comments:

The account of mathematical space as an abstraction from physical fields of force seems not to compel one to conclude that the concept of space is wholly derived from contingent facts, for the *datum* "a field of force" itself presupposes extension (field).

With regard to time, why should the chance character of a series of events render time fragmentary? The continuity of time is more closely bound up with the memory and anticipation of a mind than with the absence of causal connection between non-mental happenings.

The term "metaphysics" occurs throughout the text in the post-Kantian sense of an unscientific claim to know what cannot be experienced. "Metaphysics" thus means, in effect, "mystery." But in the uncontroversial sense of the word—a general view of the nature of things, M. Vendryès' point of view would ordinarily be called a naturalistic type of metaphysics. It is evidently not a positivistic position, but one in which the real natures of things are believed to be knowable.

The discussion of the relation of cause and probability in the natural sciences is solidly based on the actualities of physiology, genetics and thermodynamics. But as an application of the theory of probability to psychology and ethics and problems of history is outlined, it becomes clear that the probability account of human freedom almost unavoidably takes on a negative character, because of the analogy it bears to "objective probability" based on the relationship of independence among physical events. Freedom comes to mean freedom from causal connections, freedom from predictabilities, abstract freedom. One asks whether the concrete reliabilities of freedom, as embodied in personal character or in historical institutions and movements, could be adequately brought within the compass of a probabilistic humanism. The existential solidity of freedom seems to be dropped out of the account.

These, and other possible comments, I have no doubt, touch details of the argument, however. The work, taken as a whole, is surely an original and refreshing and felicitously expressed essay in the philosophy of science.

RICHARD HOCKING, Chicago, Illinois.

**Methods of Vitamin Assay.** By THE ASSOCIATION OF VITAMIN CHEMISTS, INC., Chicago, Illinois. Interscience Publishers, Inc., New York, N. Y., 1947. xviii + 189 pp. Price \$3.50.

This book, when considered from the standpoint of its scope and purpose, is a valuable and well planned venture. Its emphasis is entirely in keeping with the fact

that it was prepared by "The Association of Vitamin Chemists, Inc.," the leaders of which are almost without exception commercial chemists interested in food stuffs and pharmaceuticals, rather than investigators intent on discovering new vitamins or elucidating the functions of those already recognized.

The general scope of the volume is best indicated by the chapters on (1) Sampling, (2) Vitamin A, (3) Carotene, (4) Thiamine, (5) Riboflavin, (6) Niacin, (7) Ascorbic Acid, (8) Other Vitamin Methods, and (9) Use of Check Samples in Control of Vitamin Methods.

Chapter 8, with the exception of two short paragraphs, is limited to selected bibliographies dealing with the assay of vitamins D, E, and K, biotin, folic acid, *p*-aminobenzoic acid, inositol, choline, pantothenic acid, and pyridoxine. It is planned in later revisions to give material on the assay of these vitamins also.

The authors thought it necessary to present a certain amount of background material with regard to sampling and to the statistical handling of results. This is done in a satisfactory manner.

Another type of background seems to have been neglected, especially if the book is to be used by newcomers in the field of vitamin analysis. This is, for the want of a better term, the biological background. It is not made clear for example that "Vitamin A" is a biological concept, and the total vitamin A activity (biological) of a specified product can be expressed only in biological terms, not in terms of grams or milligrams. In those cases where the same biological activity is associated with several distinct chemical species, *e.g.*, vitamins A, D, E, K, and those of the B<sub>6</sub> group, the problem of assay is tremendously complicated, and anyone who aspires to be a "vitamin chemist" should face the facts, however unpleasant.

For the purposes outlined, the authors of this small volume have made a good selection of methods and have apparently been careful to include material which will be useful to analysts working in this field. A group of workers have reviewed various chapters in the book and have given the benefit of their advice, so that one can be sure that the material is reasonably authoritative and dependable. The volume will not be particularly useful for vitamin investigators for whom assay methods are primarily research tools.

One of the fundamental purposes of the Association of Vitamin Chemists involves "improving vitamin methods from the standpoint of . . . optimum correlation with the vitamin requirements of man." It may well be stressed that this necessitates fuller knowledge about the diversity of human requirements. At the present time our ability to determine the niacin content of green peas, for example, far exceeds our ability to interpret the finding in terms of human needs or benefit.

ROGER J. WILLIAMS, Austin, Texas.

On the Mechanism of Enzyme Action. XXXII. Fat and Sterol in *Fusarium lini* Bolley, *F. lycopersici*, and *F. solani* D<sub>2</sub> Purple

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Received October 14, 1947

INTRODUCTION

For many years the attention of numerous investigators has been directed to the study of formation of natural fats of both plant and animal origin. Moreover, it is known that a few molds, as, for example, members of the family of *Fusarium*, produce large amounts of fat when grown on various hexose-, pentose-, etc., containing media (1, 2, 3, 4). It is on this genus that our efforts have been focused.

Previous investigations from this laboratory revealed that certain *Fusaria* possess a fungus lipase, and a fragmentary study of the chemical composition of the fat of *Fusarium graminearum* Schwabe (Fgra) had been undertaken as a basis for an understanding of its formation in the cell. The solid fatty acids of that fat had been established as consisting of palmitic acid (3). It was, therefore, suggested by Dr. Nord to amplify these previous studies by a complete investigation of the composition of the fat of *Fusarium lini* Bolley (FIB), including its unsaponifiable fraction.

The genus *Fusarium* contains powerful dehydrogenating systems (5). It has also been found that *Fusarium* pigments and related compounds, depending on their structure, are able to cause an acceleration or inhibition of the rate of these dehydrogenations (6). Consequently, determinations have been carried out on the fat and sterol content of

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<sup>1</sup> Communication No. 59. This investigation was carried out with the support of grants of the Office of Naval Research and the Rockefeller Foundation. The fructose used in this study was obtained through the courtesy of Dr. R. C. Hockett, of the Sugar Research Foundation, Inc., New York, N. Y.

FlB, a non-pigment and strong fat former, and of *Fusarium lycopersici* (Flyco), a pigment- and powerful fat-producer, when these molds were grown on varying concentrations of hexoses and pentoses.

At the same time the unsaponifiable fraction of the fat of FlB, Flyco and *Fusarium solani* D<sub>2</sub> purple (FsD) was investigated to establish whether the sterol fraction was identical in the three molds.

## EXPERIMENTAL

### *Cultures Employed*

The following *Fusaria* were used in these investigations: (1) FlB, No. 5140, obtained originally from the Biologische Reichsanstalt, Berlin-Dahlem, through Dr. H. Wollenweber. (2) Flyco, obtained through the courtesy of Dr. F. L. Wellman, U. S. Dept. Agr., Beltsville, Md., Strain No. R-5-6. (3) FsD, obtained through the courtesy of Dr. William C. Snyder, Division of Plant Pathology, Univ. of California, Berkeley 4, Calif. Stock cultures of the FlB and Flyco were maintained on the usual nutrient medium (7). The FsD was kept on a modified Czapek-Dox medium consisting of:

Glucose.....	83.5 g.
NaNO <sub>3</sub> .....	8.35 g.
KH <sub>2</sub> PO <sub>4</sub> .....	8.35 g.
MgSO <sub>4</sub> ·7H <sub>2</sub> O....	0.84 g.
FeSO <sub>4</sub> .....	0.025 g.
ZnCl <sub>2</sub> .....	0.007 g.
Distilled water to	1000 ml.
pH.....	4.2

All cultures were transferred at one month intervals and periodically examined to check their purity.

### *Procedures*

The experiments were conducted by growing the *Fusaria* in 3-l. Fernbach flasks, each flask containing one l. of nutrient medium. These media were sterilized at 15 pounds pressure for 25 minutes at 120°C. and all cultures were grown at a temperature of 28–30°C. under aerobic conditions. The procedure used when preparing spore-mycelial suspensions of the microorganism for the inoculation of stock cultures and experiments was the same as that employed previously in related investigations in this laboratory (8, 9). All experiments were inoculated with 5 ml. of spore-mycelial suspension of the mold.

The time of incubation in all cases was 3 weeks, at the end of which period the mats were filtered off, washed thoroughly with distilled water, and dried in air with the aid of an electric fan. The dry mats were then ground to 40 mesh in an appropriate mill and subjected to further investigations.

Whenever the various fractions of the fat were collected for more than one day, they were stored in the cold under nitrogen to diminish the hazard of air oxidation of the fat or fatty acids. The constants of the fat were determined according to official methods (10).

*Isolation and Analysis of the Fat of F1B*

The crude mixture of fats was obtained by growing F1B on a Raulin-Thom nutrient medium (containing 2.5% glucose) and extracting the dried and finely ground mycelium with low-boiling petroleum ether in a Soxhlet for 48 hrs. The ether was then removed *in vacuo* and the residual crude fat, consisting of a dark brown semi-liquid mass, was found to have an iodine value of 84 and a saponification number of 189.

The method of saponification of the fat was the same as that described in a previous communication (3). The crude fatty acids thus obtained were converted into lead soaps according to the procedure outlined by Twitchell (11, 12), and thus separated into their solid and liquid constituents. The acids, after separation in this manner, were found to consist of 23.3% solid and 69.8% liquid acids, with palmitic acid, identified as the free acid (3), constituting 60% of the solid acids, or 19.8% of the total fatty acids.

The solution of liquid acids was placed in a distilling flask and the alcohol removed *in vacuo*. The resulting yellow oil was dissolved in ether, shaken with 6 *N* HCl to decompose any lead salts present, and the ether mixture filtered, washed with dilute NaHCO<sub>3</sub> and then with H<sub>2</sub>O. The ethereal solution was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and the ether removed *in vacuo*.

The free liquid acids were then divided into their components by fractional crystallization of their bromine addition compounds (13, 13a). Bromination of the acids was carried out in dry ether, when failure of a precipitate to appear after completion of the reaction indicated the absence of linolenic acid in the mixture. The brominated mixture was washed with three 100 ml. portions of 2% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> in a separatory funnel to remove excess bromine, and then twice with H<sub>2</sub>O. The ethereal solution was later dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After drying, the solution was filtered and the ether removed *in vacuo*. The resulting solid and oil were boiled under reflux with petroleum ether (B.P. 30–60°C.) for half an hour. On cooling, the formation of a precipitate indicated the possible presence of linoleic acid. The crystals obtained after filtration and recrystallization from a 1:1 mixture of petroleum ether (low boiling) and CCl<sub>4</sub> had a m.p. of 115–118°C. The mother liquor was concentrated to half its volume and, on cooling, a second crop of the tetrabromide was obtained and recrystallized.

This compound was subjected to a micro analysis which gave the following results: Calculated for linoleic tetrabromide: C, 36.00; H, 5.33. Found: C, 36.25; H, 5.47. This proved the presence of linoleic acid.

The petroleum ether was removed from the mother liquor *in vacuo* yielding a yellow oil, possibly the dibromide of oleic acid. To establish further the presence of oleic acid *via* a solid derivative, the dibromide was converted to the methyl ester of the acid according to the method of Rollet (14). The ester was then saponified with 4% alcoholic KOH, and the free acid obtained was converted to the dihydroxy derivative

as described by LeSueur (15). The shiny white crystals obtained had a m.p. of 130.5–132°C. Analysis: Calculated for dihydroxyoleic acid: C, 68.35; H, 11.39. Found: C, 68.50; H, 11.22. Therefore, the presence of oleic acid was established.

To determine the quantities of these two acids present in the *Fusarium* fat, thiocyanogen values and iodine numbers were run on a sample of the liquid acids. These amounted to 93 and 133, respectively. The constants indicate that the liquid acids consist of 47.1% linoleic and 53.5% oleic acid. Consequently, the total fatty acids of the fat consist of: palmitic acid: 19.8%, linoleic acid: 32.9%, oleic acid: 37.3%, and the total acids accounted for amount to 90.0%.

#### *Unsaponifiable Matter of Fusarium Fats*

Since it was observed during the above study that the unsaponifiable fraction of the fat gave a strong Liebermann-Burchard reaction, attempts were made to isolate the compound responsible for the positive test.

The unsaponifiable material, orange in color, was dissolved in 95% alcohol and treated with Norit for 10 minutes under a reflux. The solution was filtered while hot and the filtrate allowed to cool slowly to room temperature, at which point shiny crystals were obtained having a m.p. of 150–152°C. After 3 recrystallizations from absolute alcohol the white crystals had a m.p. of 158°C. From the physical and chemical properties of the compound and its derivatives it is concluded that the substance is ergosterol (16).

That the compound is a single entity was shown by preparation of the digitonide and the acetate. The former was hydrolyzed in pyridine, and recrystallization of the resulting compound, after removal of digitonin, gave crystals having a melting point of 158°C. A mixed melting point of these crystals with the original compound showed no depression. Saponification of the acetate, and recrystallization of the substance obtained after hydrolysis, showed the same characteristics.

A compound, chemically identical with the sterol described, has also been isolated from the unsaponifiable matter of the fat of Flyco and FsD. However, due to the presence of pigments in the fat of these two molds (2), the compound can be obtained and purified only with great difficulty.

## FAT AND STEROL CONTENT OF FIB AND FLYCO

Although there are numerous references in the literature attesting the occurrence of sterols in ergot, yeasts, mushrooms, and higher fungi, only a few studies have been reported which deal with the presence of sterols in molds (17, 18, 19, 20, 21). With regard to *Fusaria*, our data represent the first substantiation of the chemical identity of the sterol produced by these molds.

Peterson *et al.* stated that the sterol content of the molds tested by them varied not only with the species, but also with different strains of the same species, and that increasing the glucose content of the medium caused a decrease in the percentage of sterol present in the dry mat (17). It was interesting to us to study the effect of varying the concentration and *type* of carbohydrate in the medium on the fat *and* sterol formation in the mats of FIB and Flyco. The basic medium used was Raulin-Thom's and each series of experiments presented was run in triplicate.

*Procedure for Total Lipid Determination*

Previous investigators have noted that incomplete lipid extraction of a dry micro-organism is obtained by employing either ethyl ether or petroleum ether as the extracting solvent (22). Having experienced the same difficulty in the extraction of the dry *Fusarium* mats, it has been found that chloroform can be employed with advantage. The well-washed and finely-ground mats were air-dried, then dried over  $\text{CaCl}_2$ , and extracted with redistilled chloroform for 24 hrs. in a Soxhlet. All the substances extractable by the solvent have been termed "Total Lipid." The extract was then dried over anhydrous  $\text{Na}_2\text{SO}_4$ , quantitatively filtered through a sintered glass filter, and the filtrate made up to 100 ml., of which 25 ml. were removed for sterol determination. The remainder was placed in a dry, previously weighed beaker. The chloroform was evaporated off slowly at  $60^\circ\text{C}$ . and the beaker then placed in an oven at  $100^\circ\text{C}$ . for 30 minutes. The container was then cooled to room temperature and weighed. From the values obtained, the total lipid content of the mats was determined.

*Procedure for Total Sterol Determination*

The total sterol was determined colorimetrically (23, 24), applying several modifications and using an Evelyn photoelectric colorimeter. This method was chosen over the long and tedious digitonin method, since recovery tests conducted colorimetrically indicated that about 98% of the sterol extracted was responding to the color test with no interference by other colored substances present.

The isolated ergosterol, recrystallized 5 times, was used as the standard. It was found that maximum color development occurred within 5 minutes after addition of the last reagent and that maximum color absorption took place at 660 m $\mu$ . Readings were made on a total volume of 10 ml. This amount included a portion of the



sample to be analyzed (*e.g.*, 0.1 ml.), 2 ml. of acetic anhydride, 1 ml. of glacial acetic acid, 0.1 ml. of concentrated sulphuric acid, and enough redistilled chloroform to make a total volume of 10 ml., the glacial acetic acid being added to stabilize the color developed.

The results of these experiments are presented in Tables I and II.

In Table I the amounts of fat and sterol recorded are greater on 2.5% and 5% glucose when tap water is used than when distilled water is employed. This can be easily understood in the latter case as due to a lack of certain trace elements required for the action of certain enzymes present.

Data in Table I show that with FIB, in the case of glucose and fructose, fat and sterol contents diminish with increasing concentration of the sugar used, while with xylose both increase with rising concentration. However, in Table II, with Flyco, the results recorded are somewhat different. In all cases the amount of total lipids increases with rising concentration of sugar, while the sterol content follows the opposite course, showing an initial tendency to do so starting with 10% glucose.

The results indicate also that xylose is a much more suitable substrate for conversion into lipids than the hexoses, since both organisms

TABLE I  
*Fat and Sterol Content in Fusarium lini Bolley (FIB)*

Carbohydrate	Soln.	Average mat weight	Total lipid	Sterol
	<i>Per cent</i>	<i>g.</i>	<i>Per cent</i>	<i>Per cent</i>
Glucose <sup>a</sup>	2.5	4.78	7.1	0.88
	5	4.97	5.4	0.55
	10	4.53	4.4	0.41
Glucose <sup>b</sup>	2.5	4.28	9.7	0.99
	5	4.33	7.3	0.74
	10	4.43	4.1	0.40
Fructose <sup>b</sup>	2.5	2.33	5.8	0.75
	5	4.32	5.2	0.69
	10	5.04	5.1	0.61
Xylose <sup>b</sup>	2.5	1.23	5.2	0.44
	5	2.78	5.5	0.54
	10	2.84	6.4	0.62

<sup>a</sup> Dissolved in distilled water.

<sup>b</sup> Dissolved in tap water.

TABLE II  
*Fat and Sterol Content in Fusarium lycopersici (Flyco)*

Carbohydrate	Soln.	Average mat weight	Total lipid	Sterol
	<i>Per cent</i>	<i>g.</i>	<i>Per cent</i>	<i>Per cent</i>
Glucose <sup>a</sup>	2.5	9.83	4.9	0.49
	5	12.50	17.2	0.56
	10	13.32	19.4	0.56
Fructose <sup>a</sup>	2.5	10.50	7.8	0.49
	5	15.60	19.4	0.41
	10	20.50	26.6	0.37
Xylose <sup>a</sup>	2.5	11.72	5.7	0.48
	5	16.68	16.2	0.42
	10	18.23	30.7	0.35

<sup>a</sup> Dissolved in tap water.

are unaffected by the increasing pentose concentrations. This may be due to the fact that twice as much acetaldehyde is obtainable from the hexoses in comparison with that derived from the same amount of xylose, and that the enzymatic synthesis of fat and sterol from the aldehyde (or acetic acid), no doubt, progresses immeasurably slower than the decarboxylation of pyruvic acid to the aldehyde.

FLB produces no pigment when grown on a Raulin-Thom medium while Flyco produces a purple substance (2). It has been observed that the amount of pigment produced by the latter increases on increasing the concentration of glucose, fructose or xylose used as the substrate. Since both molds are fat formers and only one a pigment producer, it is conceivable that the function of the pigment is interrelated with the carbohydrate-fat metabolism of the mold. This may appear plausible when it is assumed that acetaldehyde, obtainable in different amounts from all three carbohydrates, is the basic building stone for fat and sterol formation, and knowing that pigments can influence the rate of certain enzymatic processes (6).

### SUMMARY

1. Palmitic, linoleic and oleic acids constitute 90.0% of the total fatty acids present in the fat of *Fusarium lini* B.

2. Ergosterol has been isolated from the unsaponifiable fraction of the fat of *F. lini* B., *Fusarium lycopersici*, and *Fusarium solani* D<sub>2</sub> purple. The compound is a single entity and is chemically identical in the three molds. FIB is capable of producing the compound to the extent of 1% of its dry mycelial weight.

3. The fat and sterol content of the dry mats of FIB and Flyco vary with varying concentration and type of sugar employed in the medium. The differences observed between the two molds may be due to the absence of pigment in the former and its interaction in the metabolism of the latter.

4. These molds prefer xylose to hexoses for conversion into fats.

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# The Enzyme Activity and Nitrogen Content of Bacterial Cells<sup>1</sup>

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Received October 28, 1947

## INTRODUCTION

From our present knowledge of the quantities of enzymes in cells, it is justifiable to assume that practically all the protein in the cells of microorganisms consists of enzyme protein ("living protein"), and that the old idea of the occurrence of enzymes only here and there in the protoplasm of the cell does not hold true. One of us paid particular attention to this fact some years ago (1). For instance, the uptake of nutritional substances by cells was discussed from this point of view.

In the present paper we shall deal with the dependence of enzyme formations upon each other in the light of the above concept. Presuming that the protein of the cell is composed of enzyme protein, the question arises as to what happens when the protein content of the cell drops considerably because of an insufficient nitrogen supply. Does the activity of all the enzymes diminish to approximately the same extent, or does the decrease affect certain enzymes only?

Virtanen and Winter (2) had noticed earlier that *B. coli* grown in a rich nutrient solution containing mineral salts, peptones, and sugar, has almost the same catalase activity as when grown in a very simple solution with ammonium salts as N-source and glycerol as C-source. Thus, the catalase content of the cells seemed to be largely independent of the quality of nutrition. The nitrogen content of the cells was, however, not determined in these experiments, which, therefore will not answer the present question.

We have now particularly examined the effect of the protein content of the cell on the activity of the adaptive and constitutive enzymes.

<sup>1</sup> Dedicated to Professor Carl Neuberg on his 70th birthday, although unfortunately delayed.

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On the supposition that the cell protein is practically all enzyme protein, it can be postulated that the formation of an adaptive enzyme (or a great increase in its enzymatic activity) effected by the respective substrate is ascribable to the formation of the particular enzyme protein from some other enzyme protein in the cell. It can then be assumed that the formation of a new enzyme causes weakening in the activity of another enzyme, preferably of a closely related type (*e.g.*, different carbohydrases). Thus, a kind of competition should exist in the formation of enzyme proteins. Virtanen and Jorma (3) were able to prove that *B. coli*, grown in a nutrient solution containing saccharose, lactose, and maltose, first ferments chiefly saccharose, and only after this has been used up, is the fermentation of lactose and maltose accelerated. It was also proved that, in a solution containing all three sugars, the bacterium has a much weaker lactase and maltase activity than when grown in a nutrient solution with either lactose or maltose alone.

The enzymes of the cell can be divided into two groups according to their importance: (a) the indispensable enzymes, and (b) those which can be dispensed with in presence of certain nutrilites without interfering with the cell activities. The former are as a rule constitutive, whereas the latter can be and often are adaptive. To the latter group belong particularly the enzymes which first attack the nutrients of the cell (*e. g.*, hydrolases). For example, the cell can exist with glucose without saccharase, lactase, maltase, *etc.*, but the presence of these enzymes in the cell greatly promotes the ability of the cell to live on different nutrilites.

As a working hypothesis we have presumed that the lowering of the protein content of the cell causes decrease primarily in the quantity of the dispensable enzymes, because these do not essentially affect the metabolism of the cell, whereas the indispensable enzymes would, to a great extent, maintain their activity. In the present work on *B. coli* we therefore chose saccharase on one hand, and catalase on the other, as the objects of the investigation. The activity of saccharase is greatly increased in solutions containing saccharose and it must, therefore, be regarded as an adaptive enzyme. The saccharase activity (*I*<sub>f</sub>) of the bacteria grown in glucose solutions is, according to the determinations of Virtanen and Winkler (4), only about 3% of the value found for bacteria grown with saccharose. Catalase is a typical constitutive enzyme (2).

## METHODS

*B. coli* (strain K3) was employed. The media for mass culture were prepared according to Karström (5) (25 g.  $K_2HPO_4$ , 25 g. saccharose, 10 g. NaCl, 100 mg.  $MgSO_4$ ,  $H_2O$  to 5 l.), except for the amount of  $(NH_4)_2SO_4$ , which varied from 8 g. to 50 mg./5 l. For preventing losses of  $NH_3$  during boiling and sterilization, the  $(NH_4)_2SO_4$  solutions were sterilized separately and added to the main solution just before inoculation. For the same reason, and to prevent losses during cultivation, the pH was kept at 6.5. Bacteria were mostly grown for 36–40 hrs. at  $37^\circ C.$ , although other culture times and temperatures were also used.

The nitrogen content of the nutrient solution greatly affects the protein content of the cells. According to our preliminary experiments, the protein content of the cells remained fairly constant if the nutrient solution contained at least 1 g.  $(NH_4)_2SO_4$ /5 l. At a lower concentration of ammonium salts the protein content of the cells decreased. In every experiment 2, 4 or 6 cultures, differing only in the ammonium sulfate content of the medium, were examined. The bacteria were centrifuged, washed

TABLE I

Each experiment comprised 6 flasks, 1.5 l. nutrient solution per flask. Quantity of  $(NH_4)_2SO_4$  in different flasks appears in the table. Duration of culture: 40 hr.; temperature  $37^\circ C.$

$(NH_4)_2SO_4$ g./5 l. nutri- ent solution	Yield of dry bacteria	N-content of dry bacteria	<i>Kat.f.</i> $10^4$	<i>Kat.f.N</i> $10^3$	<i>I/f</i> $10^4$	<i>I/f N</i> $10^3$
	<i>mg.</i>	<i>Per cent</i>				
Expt. 26						
0.05	15.0	8.8	4.5	5.1	<60	<60
0.1	17.8	9.3	3.6	3.9	<60	<60
0.5	117.3	10.3	5.1	5.0	120	116
1.0	381.5	12.6	5.6	4.5	500	392
2.0	475.0	12.4	4.1	3.3	648	520
8	408.5	12.7	2.7	2.1	428	340
Expt. 36						
0.1	24.0	10.4	7.7	7.4	88	84
0.5	153.8	11.6	8.1	7.0	172	148
1.0	340.5	12.3	8.0	6.6	544	448
3.0	279.8	11.9	5.8	4.9	512	432
5.0	358.3	12.7	5.7	4.5	464	364
8.0	409.3	12.8	5.7	4.4	532	416
Expt. 35						
0.1	21.3	6.5	2.3	3.5	128	196
0.5	88.5	9.3	4.2	4.2	104	100
1.0	170.8	11.9	7.8	6.6	356	300
3.0	257.8	12.1	8.4	6.9	576	476
5.0	207.8	12.9	5.0	3.8	596	460
8.0	219.5	12.5	7.2	5.8	564	448

and resuspended in distilled water. From this suspension samples were taken for various estimations. Determinations of the protein content were based on the total N-content according to the Kjeldahl method. The dry weight of the samples was obtained by drying at 95–105°C. for 4 days. Catalase activity was determined by permanganate titration [cf. Virtanen and Karström (5); Karström (7)] immediately after centrifugation, and saccharase by using the Bertrand method in a 40-hr. old autolyzate under toluene at pH 7. From the  $k$  values obtained, the activity was calculated/g. dry bacterial mass, and expressed, according to v.Euler, as  $Kat.f.$  and  $If$ . In addition, the activity was calculated/g. nitrogen and then expressed as  $Kat.f.N$  and  $If N$ .

## RESULTS

Table I gives the yield of bacteria, nitrogen content of the bacterial mass, and the catalase and saccharase activities in nutrient solutions with varying N-content.

## DISCUSSION

In examining the results presented in Table I and Figs. 1 and 2 we can note the following.

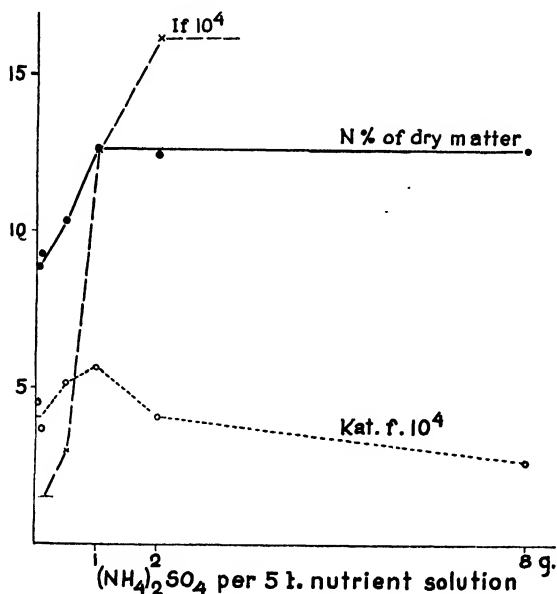


FIG. 1.\* Catalase and saccharase activity ( $Kat.f.$  and  $If$ ) of *B. coli* cells with varying N-contents. The N-content was decreased by cultivating the bacteria in nutrient solutions with low  $(NH_4)_2SO_4$  concentration (cf. Exp. 26, Table I).

\* In Figs. 1 and 2  $If$  values have been divided by 40 to fit all curves to the same scale.

The N-content of the cells remains fairly constant when the  $(\text{NH}_4)_2\text{SO}_4$  content exceeds 1 g./5 l. of nutrient solution (over 40 mg. N/l.). Slight discrepancies can be noted in this respect between different experiments, but a quantity of 2 g.  $(\text{NH}_4)_2\text{SO}_4$ /5 l. regularly produces maximum N-content in the cells under the conditions prevailing in our experiments. As a rule, the N-content of *B. coli* is very high, 12–13% of the dry weight of the cells, with adequate nitrogen supply. When the amount of  $(\text{NH}_4)_2\text{SO}_4$  falls below 1 g./5 l., the N-content of the cells

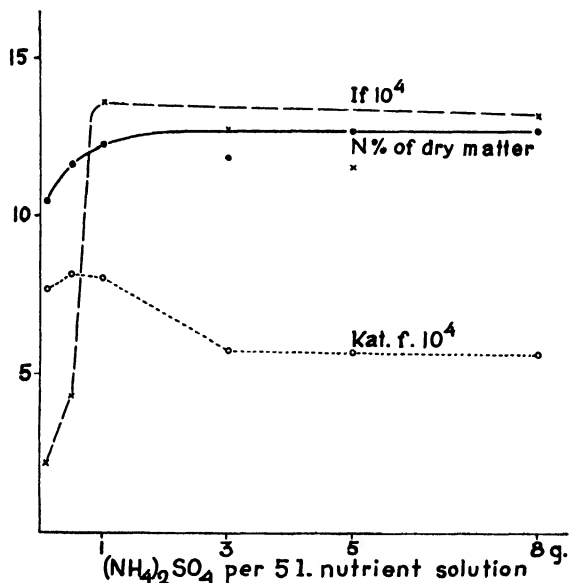


FIG. 2.\* Catalase and saccharase activities (*Kat.f.* and *If*) of *B. coli* cells with varying N-contents (*cf.* Exp. 36, Table I).

rapidly decreases. In the worst case the N-content lowered to 6.5% of the dry matter, or half the maximum. The nutrient solution then contained 0.1 g.  $(\text{NH}_4)_2\text{SO}_4$ /5 l., or 4 mg. N/l.

As soon as the N-content of the cells decreases slightly, their saccharase activity abruptly diminishes (Figs. 1 and 2). In cells with lower nitrogen content the *If* is only about 10–15% of the maximum. In certain experiments the *If* has been too low for determination. The decrease in saccharase activity was regularly much more abrupt than that in nitrogen content.



The catalase activity of the cells, on the other hand, depends very little on their nitrogen content. True, a slight fall is also caused in the *Kat.f.* by the drop in the N-content of the cells, but only in a single case, when the N-content of the cells was exceptionally lowered to 6.5% of the dry matter, was the fall greater. *Kat.f.* calculated on a nitrogen basis was not decreased nearly as much as when calculated to dry matter. The values of *Kat.f.N* are of great significance in indicating the relationship of the enzymatic activity and the protein content of the cells. In all our experiments the *Kat.f.* was a little higher in cells grown in nutrient solution with 1–3 g.  $(\text{NH}_4)_2\text{SO}_4/5$  l. than in cells grown with larger or smaller amounts of nitrogen. It is difficult to explain this slight maximum while the N-compounds of the cells are not sufficiently well analyzed. We shall later take up this question in detail. It is noteworthy that the values of *Kat.f.N* do not in all cases indicate such a maximum. In all cases, our results show definitely that the catalase activity of *B. coli* is largely independent, over a wide range of ammonium sulfate concentrations, of the amounts of ammonium salts in the nutrient solution and of the nitrogen content of the cells.

Accordingly, the constitutive catalase enzyme indispensable for metabolism is well preserved in the cell, even when the nitrogen nutrition is so poor that a weak growth is only just possible and the protein content is considerably decreased. On the other hand, the adaptive dispensable saccharase enzyme decreases considerably or disappears almost entirely if the bacteria are cultivated in low concentrations of ammonium salts. Because saccharase improves the living conditions of bacteria, making growth possible even when saccharose forms the only carbon source, cultivation of bacteria in solutions with a very low nitrogen content causes production of cells with quite different living conditions from those supplied with an abundant nitrogen supply. Seemingly, the low-protein *B. coli* bacteria belong to a different type than those richer in protein, since they are no longer able to utilize the same nutrients as the latter. This result shows what an enormous effect nitrogen nutrition has upon the enzymatic activity of the cells.

The results are in agreement with the working hypothesis mentioned at the beginning of this paper. The catalase activity of the low-protein cells has remained practically unchanged, whereas the ability of the cells to form saccharase in saccharose solutions has practically disappeared in the extreme cases. Lowering of the protein content thus

causes an enormous decrease or loss of the dispensable enzymes, probably because there will not be enough protein to serve as building material for these enzymes.

The adaptive formation of the enzymes enables the cell to utilize its protein to the greatest possible advantage since, if necessary, the cell may thus gain new activities through transformation of some enzyme protein to building material for a new enzyme. Since the adaptive enzyme formation is a hereditary property, it is likely that the cell always contains a certain amount of adaptive enzymes, too, although they cannot often be detected owing to their small amount. They may function as centers when the adaptive enzyme is being formed in the presence of the particular substrate. The proteins of closely related enzymes may be assumed to change to proteins of these new enzymes.

In the foregoing we have started with the idea that the quantity of the protein part of the enzyme primarily determines the activity of the enzyme in the cells; in other words, the protein part is the minimum factor. This may be true in most cases because just the protein of the enzyme dictates its specificity. Spiegelman *et al.* (8) have recently shown that the adaptation of yeast to ferment galactose involves the formation of an apoenzyme. This was previously suggested by the earlier observations of v. Euler and Jansson (9). It is, of course, likewise possible that the coenzyme part is, in certain cases, the minimum factor. This evidently applies to cells which require certain growth factors in their nutrition.

The hypothesis that the proteins of the cell are practically enzyme proteins involves the possibility that the cell may also contain some enzymatically inactive protein (reserve protein). This may be used for the formation of new enzymes. For the sake of simplicity we have, in this paper, based the hypothesis on the assumption that the enzyme proteins are transformed to each other, although conversion of inactive protein to enzyme proteins may also be possible.

### SUMMARY

It has been shown that, when *B. coli* is grown in varying concentrations of ammonium sulfate, the nitrogen content of the cells is lowered. There is a fall in the N-content as soon as the ammonium salt concentration reaches a definite limit. Under the conditions used, the limit was about 40 mg. N/l. The nitrogen content of the cells was in the utmost cases decreased from about 13% to 6.5% of dry matter. The saccharase activity of the cells rapidly diminished with decrease

in the protein content of the cells. The abrupt fall in the saccharase activity started immediately after the N-content of the cells fell below 12%. When the N-content fell to about 10%, *If* was only 10–20% of the maximum or even less. The catalase activity, on the other hand, depended but slightly on the N-content of the cells. The results are discussed in the paper.

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# Mexican Turtle Oils. I

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Received April 30, 1947

## INTRODUCTION

Empirical use of turtle oils, with diverse therapeutic applications, is well known in various places. In the chronicle of his second voyage, Christopher Columbus states that the Cape Verde Islands were extensively frequented by wealthy Portuguese who came there to have their leprosy cured with turtle oil from the Isles. In Mexico particularly, turtle oils are greatly esteemed by the Indians in various sections of the country for the treatment of leprosy, tuberculosis and other affections of the chest. Most of these data have been gathered in Mexican publications (1, 2). The Indian custom of utilizing these oils is so widespread that President Alvaro Obregón issued an official decree encouraging their use for therapeutic purposes. Scientific experiments were carried on at the Pasteur Institute in Paris, and good results were obtained in the treatment of experimental leprosy in rats (3) with turtle oils obtained from Mexico (green turtle oil), but without specifying the animal species to which those turtles belonged; the tests could not be completed because of lack of material (4). Finally the widespread use of turtle oils in cosmetics because of their beneficial effect on the skin is also well known.

All this influenced us to undertake the systematic study of Mexican turtle oils. The classification of the animals by Dr. Rafael Martín del Campo of the Biological Institute of the University of Mexico is gratefully acknowledged.

## EXPERIMENTAL

Up to the present, we have been able to secure oils from the following species, all of which are edible. For various reasons, the available amounts of each oil sample were quite variable and the analyses had to be carried on accordingly.

*Dermatemys mawii*, an earth or sweet water turtle, known as "tortuga blanca" (white turtle), obtained at Puerto de Alvarado (Veracruz). Length of shell is 28-30 cm.

*Chelone mydas* Linn., a sea turtle, called "tortuga verde" (green turtle) or "golfina," but also termed "cahuama" in certain places. The oil tested at the Pasteur Institute probably came from this type. Oil from two specimens was obtained at two different seasons, but from the same general location (Pacific coast); both were males, weighing about 50 kg. and having a shell about 50 cm. in length. One was caught in Abuya, north of Mazatlan (Sinaloa) during the summer (June), and the other in Acapulco (Guerrero) during the winter (December). The summer specimen yielded a large quantity of oil ( $\pm 2$  l.), whereas the winter specimen yielded much less ( $\pm 150$  cc.), both having approximately the same weight.

*Lepidochelis olivacea* Esch., a sea turtle, called "tortuga oscura" (dark turtle) which is caught in May in Mazatlan. It was a female weighing  $\pm 45$  kg. with a 60 cm. shell, which gave but little oil (0.5 l.) and had a large quantity of eggs.

*Caretta caretta* Linn., a sea turtle of the Atlantic, called "tortuga tonta" (dull turtle) and "cahuama." They usually weigh 200 kg. and have a shell 90 cm. in length. The oil commercially sold in Mexico City is from this species, and comes from the island of Mujeres, off the Caribbean coast of Yucatan.

TABLE I  
Constants of Mexican Turtle Oils

	<i>Dermatemys mawii</i>	<i>Caretta caretta</i>	<i>Chelone mydas</i>		<i>Lepidochelis olivacea</i>
			Abuya (summer)	Acapulco (winter)	
Melting point	15°C.	30-34°C.	23-26°C.	17-20°C.	25-27°C.
Density (20°C.)	0.9218	0.9281	0.965	—	0.9301
Index of refraction (40°C.)	1.4560	1.4634	1.4580	—	1.4582
Acid number	0	14.0	0	2.1	0
Saponification number	183.0	189.0	198.4	183.5	181.6
Iodine number (Hanus)	83.0	76.6	73.5	89.2	93.3
Total acids (per cent)	84.0	90.0	93.5	89.4	89.0
Unsaponifiable material (per cent)	—	3.0	0.84	2.2	1.86

All oils are extracted by slowly rendering the adipose panicle. They are liquids which on standing in the cold deposit varying amounts of a white solid fat, but are all liquids above 35°C. Their most significant constants are given in Table I

Considering the use of these oils in the treatment of leprosy and tuberculosis, it was of interest to determine the possible presence of branched-chain acids of the types found in Chaulmoogra oil or those present in the *Bacillus tuberculosis*. Both types of substance are identified by their optical activity. All turtle oils were carefully ex-

aminated in the polarimeter, but no rotation was observed in any case, either with the whole oils or with the various acid fractions. None of the acids contains nitrogen, sulfur or phosphorus. None of the turtle oils extracted from the adipose panicle contains Vitamin A, but those extracted from the liver do contain this substance.

In subsequent papers, an account is given on the composition of the acids present in the fat.

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## Mexican Turtle Oils. II. *Chelone mydas*, Linn.

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Received April 30, 1947

Analyses have been made of the oil of two specimens of *Chelone mydas* from the Pacific coast in Mexico, the detailed procedure being given elsewhere (1). The usual method was followed: the bulk was saponified, the unsaponifiable fraction extracted with ether, the total acids separated as their lead salts, and the methyl esters distilled *in vacuo* (2).

TABLE I  
*Distillation of the Solid Methyl Esters from the Summer Fat*

Fraction	Amount	Saponification equivalent	Iodine number [Hanus]
	g.		
1	9.23	226.5	4.5
2	6.32	229.5	4.7
3	6.55	248.5	5.2
4	10.90	255.9	5.5
5	11.65	263.9	5.8
6	13.49	269.8	5.9
7	15.41	272.2	6.4
8	14.40	277.2	6.8
9	16.80	280.2	6.9
10	16.31	287.0	6.9
11	11.30	292.5	7.0
12	2.51	304.0	7.4
13	16.00	309.7	11.3
	150.87		

### EXPERIMENTAL

*Specimen from Abuya (Summer).* 550 g. of fat gave 514 g. of total acids (93.5%). From the insoluble lead salts 154 g. of "solid" acids (30.5%) were obtained, and 350 g.



TABLE II  
*Distillation of the Liquid Methyl Esters from the Summer Fat*

Fraction	Amount	Saponification equivalent	Iodine number [Hanus]
	<i>g.</i>		
1	2.87	207.5	13.2
2	4.16	220.2	16.4
3	4.66	231.4	19.2
4	4.92	236.5	30.1
5	4.56	249.6	44.3
6	4.10	260.2	53.4
7	5.10	268.2	62.7
8	4.12	281.2	78.9
9	4.72	289.3	90.0
10	5.72	295.4	108.7
11	4.75	296.2	110.7
12	4.69	298.6	125.8
13	5.23	300.5	138.4
14	6.15	302.6	148.5
15	4.85	305.1	162.9
16	4.25	310.5	191.8
17	5.50	316.0	221.2
	80.35		

TABLE III  
*Composition of the Fatty Acids of the Summer Oil of Chelone mydas*

	"Solid" (30.5%)	"Liquid" (69.5%)	Total (Per cent acids)
Saturated:			
Capric (C <sub>10</sub> )	—	0.8	0.8
Lauric (C <sub>12</sub> )	1.8	8.4	10.2
Myristic (C <sub>14</sub> )	4.1	5.3	9.4
Palmitic (C <sub>16</sub> )	13.8	3.4	17.2
Stearic (C <sub>18</sub> )	7.0	—	7.0
Arachidic (C <sub>20</sub> )	1.4	—	1.4
Unsaturated:			
C <sub>14</sub> (—2.0 H)	—	0.9	0.9
C <sub>16</sub> (—2.0 H)	—	9.9	9.9
C <sub>18</sub>	2.4	30.0	32.4
	(—2.0 H)	(—2.6 H)	(—2.6 H)
C <sub>20</sub>	—	10.8	10.8 <sup>†</sup>
		(—6.2 H)	(—6.2 H)

of liquid acids (69.5%) were liberated from the soluble lead salts. The solid acids were esterified completely and yielded 153 g. of methyl esters which were distilled at 2 mm. in a Willstätter flask (Table I). Of the liquid acids only an aliquot portion (90 g.) was esterified, yielding 85 g. of methyl esters, distillation of which, carried out under identical conditions, is recorded in Table II. The acids, as well as the "liquid" esters, were handled and kept in an inert atmosphere. The "liquid" acids give only very limited amounts of bromides insoluble in ethyl ether, but give a large precipitate in petrol ether, indicating a small proportion of triene acids and a large amount of dienes.

Calculation of the data obtained in Tables I and II gives the composition in fatty acids indicated in Table III.

*Specimen from Acapulco (Winter).* 89.5 g. of fat gave 80.0 g. total acids (89.4%) which were separated into 25.5 g. of "solid" acids (33.1%) and 51.5 g. of "liquid" acids (66.9%) by means of their lead salts. These were converted into their methyl esters yielding 25.0 and 52.0 g., respectively. The products of their fractional distillation from a Willstätter flask at 2 mm. pressure are included in Tables IV and V. Table VI gives the composition calculated from the data of both tables.

TABLE IV  
*Distillation of the "Solid" Methyl Esters of the Winter Fat*

Fraction	Amount	Saponification equivalent	Iodine number [Hanus]
	g.		
1	2.07	244.2	3.6
2	3.12	255.8	4.0
3	2.86	261.5	5.6
4	3.10	266.6	6.9
5	2.04	273.0	8.8
6	2.03	279.4	11.6
7	1.83	285.9	12.7
8	1.62	295.2	15.6
9	1.92	303.2	16.9
10	3.24	315.4	18.7
	23.83		

## DISCUSSION

Two bibliographies exist on the fatty acid content of the oil of the "green turtle," *Chelone mydas*, one by Green and Hilditch (3) concerning a specimen obtained from the Seychelles and the other by Ogata and Minato (4) on a specimen from New Guinea. These authors'

TABLE V  
*Distillation of "Liquid" Methyl Esters of the Winter Fat*

Fraction	Amount	Saponification equivalent	Iodine number (Hanus)
	<i>g.</i>		
1	2.85	246.6	71.3
2	2.89	252.1	77.1
3	2.72	265.5	80.5
4	2.88	270.6	88.2
5	2.82	275.5	97.2
6	3.20	279.7	107.0
7	2.65	283.5	118.4
8	3.15	290.0	128.3
9	3.22	290.4	131.2
10	2.82	293.1	133.7
11	3.96	294.9	136.0
12	3.31	295.3	138.3
13	2.70	296.0	143.7
14	2.42	301.4	167.2
15	2.63	309.4	191.1
16	3.02	313.6	223.5
17	3.25	319.1	250.7
	50.49		

TABLE VI  
*Composition of the Fatty Acids of the Winter Oil of *Chelone mydas**

	"Solid" (33.1%)	"Liquid" (66.9%)	Total (Per cent acids)
Saturated:			
Myristic	7.0	1.2	8.2
Palmitic	13.1	3.6	16.7
Stearic	5.6	—	5.6
Arachidic	3.5	—	3.5
Unsaturated:			
C <sub>14</sub> (−2.0 H)	—	4.4	4.4
C <sub>16</sub> (−2.0 H)	—	13.6	13.6
C <sub>18</sub>	3.9	34.1	38.0
	(−2.0 H)	(−3.2 H)	(−3.1 H)
C <sub>20</sub>	—	10.0	10.0
		(−6.5 H)	(−6.5 H)

results are compared with ours in Table VII. As can be seen, the results obtained from the Abuya specimen are in fair agreement with those of both authors, the only divergence being that the quantity of unsaturated acids  $C_{20}$  is larger at the expense of the unsaturated acids  $C_{18}$  which are reported as smaller in amount and having a somewhat higher degree of unsaturation. Contrariwise, the specimen from Acapulco shows a considerable difference, the most salient fact being the complete absence of lauric acid and the small proportion of capric acid; the

TABLE VII  
*Relative Content in Fatty Acids of the Oil from Chelone mydas*

	Seychelles	New Guinea	Mexico	
			Summer	Winter
Capric	0.2	3.5	0.8	—
Lauric	13.3	14.2	10.2	—
Myristic	10.6	7.2	9.4	8.2
Palmitic	17.0	15.2	17.2	16.7
Stearic	4.1	6.8	7.0	5.6
Arachidic	—	—	1.4	3.5
Myristoleic	1.3	2.6	0.9	4.4
Palmitoleic	7.8	10.9	9.9	13.6
$C_{18}$	39.6	39.4	32.4	38.0
	(-2.2 H)	(-2.0 H)	(-2.6 H)	(-3.1 H)
$C_{20}$	6.1 (-6.3 H)	traces	10.8	10.0
			(-6.2 H)	(-6.5 H)
$C_{22}$			—	—

saturated acids are present in lesser amounts, except for arachidic acid which is present in larger quantity. On the other hand, there is an increased proportion of unsaturated acids  $C_{14}$ ,  $C_{16}$ , and  $C_{18}$  as well as in their average unsaturation. Taking into account that both Abuya and Acapulco lie on the Western coast of Mexico it is not likely that such remarkable differences should be attributed to the difference in geographic origin, in spite of the different latitudes ( $23^\circ$  and  $17^\circ$ , respectively), since there is a marked resemblance between one of these turtles and those of some species obtained in places as remote as the Seychelles and New Guinea. Hilditch (5) has pointed out that the presence of lauric acid and of large quantities of myristic acid might be

a characteristic proper to the species, not to the family; or perhaps that it depends on the food. This latter alternative seems more probable to us, if it is taken into consideration that the specimen from Abuya was caught in the summer (June) whereas that from Acapulco was taken in the winter (December). We believe that this difference in season affords a satisfactory explanation for the difference in composition. We point out here, and it will be seen later on from the analyses of other turtle species, that the presence of large quantities of lauric and myristic acid is, at the most, a special case with *Chelone mydas*, as shown by Hilditch (5), since it is absent in other sea turtles (6), and in the only known case of earth turtle. As shown in our analysis this peculiar characteristic is not constant even in *Chelone mydas*.

The different composition of the winter fat can be explained, as surmised by Hilditch (5), by the fact that the available food may be different in summer and winter, or it may also be caused by a different metabolism in the animal in the different seasons of the year. At any rate, it is to be noted that the different qualitative composition is accompanied by a notable quantitative difference: the storage fat of the turtle in the winter represents only a tenth of that stored by the same species in the summer.

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## Mexican Turtle Oils. III. *Caretta caretta*, Linn.

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Received April 30, 1947

### INTRODUCTION

The oil from the sea turtle *Caretta caretta*, abundant in the Caribbean (1), is readily available in the Mexican market, as it is widely used in cosmetics. We shall presently describe the analysis carried out for its fatty acids, following the usual technique (2).

### EXPERIMENTAL

One hundred g. of oil, saponified with alcoholic KOH after previous elimination of the unsaponifiable matter by continuous (30 h.) extraction with ether, gave 90.0 g. of total acids which were separated by means of their lead salts into 26.5 g. (42.9%) of

TABLE I  
*Distillation of "Solid" Methyl Esters*

Fraction	Amount	Saponification equivalent	Iodine number [Hanus]
	<i>g.</i>		
1	1.82	246.7	1.3
2	1.91	252.2	1.2
3	4.39	264.1	1.5
4	4.59	273.1	2.2
5	4.93	278.6	2.7
6	4.29	284.3	3.3
7	4.06	285.2	3.1
8	8.00	302.7	5.1
	33.99		

"solid" acids and 48.5 g. (57.1%) of "liquid" acids. After conversion to their methyl esters, 36 and 48 g., respectively, were obtained which were further distilled *in vacuo* from a Willstätter flask. The "solid" esters were distilled at 1 mm.; the "liquid" esters at 2 mm. The results of the fractionations are included in Tables I and II. The acids

as well as the "liquid" esters were prepared and handled in an inert atmosphere. Qualitative tests showed significant amounts of bromides insoluble in ethyl ether and petrol ether. A test carried out with another sample of total acids from 100 g. of fat did not yield appreciable amounts of steam-volatile acids (4 h. steam-distilled).

Table III indicates the fatty acid content calculated from the data in Tables I and II.

TABLE II  
*Distillation of "Liquid" Methyl Esters*

Fraction	Amount g.	Saponification equivalent	Iodine number [Hanus]
1	4.55	243.5	65.6
2	5.85	266.6	80.8
3	6.10	271.4	91.1
4	6.32	281.7	124.2
5	5.40	285.9	143.0
6	7.40	292.1	155.5
7	10.55	297.2	178.8
	46.17		

TABLE III  
*Fatty Acid Content of Oil from *Caretta caretta**

	"Solid" (42.9%)	"Liquid" (57.1%)	Total (Per cent acids)
Saturated:			
Myristic	4.5	2.1	6.6
Palmitic	19.5	2.3	21.8
Stearic	15.5	—	15.5
Arachidic	1.9	—	1.9
Unsaturated:			
C <sub>14</sub> (−2.0 H)	—	3.5	3.5
C <sub>16</sub> (−2.0 H)	—	18.0	18.0
C <sub>18</sub>	1.5	29.9	31.4
	(−2.0 H)	(−3.8 H)	(−3.7 H)
C <sub>20</sub>	—	1.3	1.3
		(−8.6 H)	(−8.6 H)

## DISCUSSION

In connection with *Chelone mydas* (3) there is a closer approximation to the "winter fat." The fat from *Caretta caretta* resembles more closely the fats of amphibia and reptiles in its content of palmitic and myristic acids and lack of the lower acids. If compared with other fats of amphibia and reptiles, the oil from *Caretta caretta* shows the highest content in stearic and palmitoleic acid and the lowest in unsaturated acids,  $C_{18}$  and  $C_{20}$ , together with the highest average unsaturation of acids  $C_{18}$ .

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## Mexican Turtle Oils. IV. *Lepidochelis olivacea* Esch.

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Received April 30, 1947

The fatty acid content of the oil from *Lepidochelis olivacea* has been determined. The specimen was a female caught on the Pacific coast of México (1).

### EXPERIMENTAL

Forty-two g. of fat, saponified with alcoholic KOH, were freed from the unsaponifiable matter by continuous extraction with ether (30 h.), yielding 37.5 g. total acids (89.0%) which were separated into "solids" and "liquids" by means of their lead salts, giving 12.0 g. (31.6%) and 25.5 g. (68.4%), respectively. After being converted into their methyl esters, they gave 11.5 g. and 25.0 g., respectively, which were distilled *in vacuo* at 2 mm. from a Willstätter flask.

The results obtained in both distillations can be seen in Tables I and II. In Table III the composition in fatty acids is given.

TABLE I  
*Distillation of the "Solid" Methyl Esters*

Fraction	Amount	Saponification equivalent	Iodine number [Hanus]
	<i>g.</i>		
1	2.55	266.8	3.5
2	2.35	270.7	6.2
3	2.47	276.1	8.8
4	1.72	281.6	12.3
5	1.78	296.3	28.5
	10.87		

TABLE II  
Distillation of the "Liquid" Methyl Esters

Fraction	Amount	Saponification equivalent	Iodine number [Hanus]
	g.		
1	3.08	267.9	57.8
2	2.82	278.1	91.7
3	2.89	288.1	122.2
4	3.17	295.1	134.8
5	3.46	300.9	150.9
6	3.15	306.8	170.7
7	3.95	309.6	172.8
	<hr/> 22.52		

TABLE III  
Fatty Acid Content of the Oil from *Lepidochelis olivacea*

	"Solid" (31.6%)	"Liquid" (68.4%)	Total (Per cent acids)
Saturated:			
Myristic	1.4	0.4	1.8
Palmitic	20.7	5.4	26.1
Stearic	5.5	---	5.5
Unsaturated:			
C <sub>16</sub> (-2.0 H)	--	11.7	11.7
C <sub>18</sub>	4.0	36.1	40.1
	(-2.0 H)	(-3.2 H)	(-3.1 H)
C <sub>20</sub>	--	14.8	14.8
		(-5.0 H)	(-5.0 H)

### DISCUSSION

The fat from *Lepidochelis olivacea* has, of all the sea turtles, the closest resemblance to the average type of fats from amphibia and reptiles (2). It has the smallest proportion of unsaturated acids C<sub>18</sub>, and the highest of palmitic acid and unsaturated acids C<sub>20</sub>. The only value that deviates notably from those of fats from amphibia and reptiles is the average unsaturation of acids C<sub>18</sub>, which is somewhat higher than usual. If compared with fats from other turtles, it comes

closer to the composition of the only known fat from earth turtle, *Testudo graeca* (3), which falls within the general type of amphibia and reptiles. If compared to sea turtles, it resembles the fat of *Chelone mydas* (4) in its content of stearic, palmitic and unsaturated acids of  $C_{20}$ , but comes closer to the composition of *Caretta caretta* (5) in its content of palmitic and myristic acids as well as in the lack of lower saturated acids. It seems, therefore, that turtles possess fats with wide variations as to their composition. In Table IV we have gathered the

TABLE IV  
*Composition of Fats from Sea Turtles (Extreme Values)*

Saturated	Capric	0 - 0.8
	Lauric	0 - 13.3
	Myristic	1.8-10.6
	Palmitic	16.7 26.1
	Stearic	4.1-15.5
	Arachidic	0 - 3.5
Unsaturated	$C_{11}$ (-2 H)	0 - 4.4
	$C_{16}$ (-2 H)	7.8-18.0
	$C_{18}$ (-2 H to -3.7 H)	31.4-40.1
	$C_{20}$ (-5 H to -8.6 H)	0 - 14.8

extreme values of the known analyses of the fats from sea turtles. We shall omit earth and fresh water turtles, in which there are biological reasons which justify a difference in composition.

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# The Metabolism of Various Phthalein Dyes by Rat Liver Slices<sup>1</sup>

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Received July 25, 1947

## INTRODUCTION

Phthalein dyes administered to animals are excreted in various ways. Di Somma (1) found that 45% of phenolphthalein given to rabbits appears in the urine as a glucuronide and only a small amount appears in the feces. Tetrachlorophenolphthalein is rapidly conjugated in the dog but is excreted in the bile and feces only (2). Phenolsulfonephthalein is used as a kidney function test and is rapidly excreted in the urine, although there is evidence that part is excreted in the bile and reabsorbed from the intestine (2). Little or none is conjugated. It appears that the sulfonic acid compounds, unlike the true phthaleins, are not readily conjugated in the body. The question arises whether this difference is due to different rates of absorption and excretion or whether the liver has difficulty conjugating phenols containing a sulfonic acid group. The metabolism of these three dyes and thymolsulfonephthalein was therefore studied *in vitro*.

## EXPERIMENTAL

Rat liver slices were suspended in 4.0 cc. of Krebs' bicarbonate solution, pH 7.4, which had been previously saturated with a 95% O<sub>2</sub>-5% CO<sub>2</sub> gas mixture. The experiments were carried out in this atmosphere in 50 cc. Erlenmeyer flasks shaken at 37°C. The dye to be tested was rubbed into a paste with 0.2 cc. of 4% NaOH, water was added to within 2.0 cc. of the desired volume, the solution neutralized with 1 *N* HCl, and then made up to volume. The solutions as finally made up were  $6.29 \times 10^{-3}$  *M* phenolphthalein,  $6.58 \times 10^{-3}$  *M* phenoltetrachlorophthalein,  $5.64 \times 10^{-4}$  *M* phenolsulfonephthalein (phenol red) and  $4.28 \times 10^{-4}$  *M* thymolsulfonephthalein (thymol blue).

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<sup>1</sup> The grant in aid for this investigation, received from the Duke University Research Council, is gratefully acknowledged.

The experiments were carried out in triplicate. To certain vessels in each experiment the dyes were added at the end of the incubation period. These served as standard controls. Tissue controls without added dye were used for the sulfonephthaleins. When either of the phenolphthaleins were used, each tube served as its own tissue control. At the end of the experiment 1.0 cc. of 20% trichloroacetic acid was added to all the vessels, allowed to act for 10 minutes and then 5 cc. of 95% ethyl alcohol added. After 10 minutes the solution was decanted, centrifuged, and the supernatant poured off and used for analysis.

After suitable dilution of the phenolphthalein and phenoltetrachlorophthalein solutions, an aliquot was estimated immediately by transferring it to an Evelyn colorimeter tube, and adding 25 cc. of water and 10 cc. of glycine buffer at pH 10.2. The tubes were then read in an Evelyn photoelectric colorimeter using No. 540 filter. This initial reading is the tissue control. To develop the red color 10% NaOH was added dropwise until no further increase in color could be obtained. Phenol red was estimated in the same manner, except that 4.0 cc. each of water and buffer were used. To estimate thymol blue, 7.0 cc. of water was added and the red color developed by the addition of concentrated HCl. The accuracy of these estimations is within 5%.

To a second aliquot of the supernatant 0.5 cc. of concentrated HCl was added and the solutions autoclaved for 2 hours at 20 lbs. After autoclaving, the solutions were washed into colorimeter tubes with three 1.0 cc. portions of water, 1.5 cc. of 95% ethyl alcohol added and the solutions made up to the required volume. The alkaline dyes were neutralized with 40% NaOH and then the color was developed and read as above. Thymol blue solutions required no further acidification.

In the following table the "amount added" value represents the amount recovered from those vessels to which the dye was added at the end of the incubation period. Since there is some loss of free dye on autoclaving the centrifugate, these control vessels were always autoclaved at the same time and under the same conditions as the experimental tubes. The amount of dye left in these control tubes is considered the "amount added" value for the autoclaved tubes.

Under the conditions of the experiment phenolphthalein and phenoltetrachlorophthalein disappear rapidly when added to liver slices. The amount that disappears varies with the concentration of the dye and the time of incubation. The effect of concentration is shown in Table I. Much less phenol red and thymol blue, however, disappear under the same conditions (Table I).

To see whether the amount of dye dissociated affected the rate of disappearance, phenol red and phenolphthalein were incubated at pH 6.7. The rates of disappearance of the two dyes were the same at pH 6.7 and 7.4. Therefore, it is the substitution of a sulfonic for a carboxylic acid and not the dissociation that is responsible for the different rates of disappearance of the two dyes.

This disappearance might be the result of oxidation or conjugation. If conjugation occurs, autoclaving the solutions in acid should result in the recovery of the dye since the free phenol group is necessary for the color. Conjugation was found to be responsible for part of the disappearance of each dye and the proportion was dependent upon the amount and type of dye present. These results are also shown in Table I. Apparently the liver is able to conjugate only a certain amount of each compound in a given length of time. When the limit is reached, another mechanism, probably oxidation, is used.

TABLE I

*The Effect of the Concentration of Phenolphthalein, Phenoltetrachlorophthalein, Phenolsulfonephthalein, and Thymolsulfonephthalein on the Percentage Conjugated by 300 mg. (Wet Weight) of Rat Liver Slices Incubated 4 hours at 37°C.*  
(The tissue controls have been subtracted.)

Compound	Amount added	Amount recovered	Per cent <sup>a</sup> disappeared	Amount added (auto-claved)	Amount recovered after auto-claving	Per cent <sup>b</sup> conjugated
	<i>mg.</i>	<i>mg.</i>		<i>mg.</i>	<i>mg.</i>	
Phenolphthalein	.204	.006	97%	.204	.207	100%
	.435	.051	88%	.402	.384	94%
	.636	.162	75%	.573	.474	77%
	.747	.261	65%	.717	.558	68%
Phenoltetrachlorophthalein	.321	.120	63%	.297	.267	84%
	.900	.507	44%	.837	.684	59%
	1.200	.759	37%	1.113	.924	54%
Phenolsulfonephthalein	.027	.014	49%	.019	.012	24%
	.053	.037	31%	.034	.025	10%
	.111	.100	10%	.105	.094	0%
	.300	.300	0%	.300	.300	0%
Thymolsulfonephthalein	.030	.019	38%	.029	.023	47%
	.062	.039	37%	.062	.047	35%
	.090	.065	29%	.090	.067	14%

$$^a \% \text{ Disappeared} = 100 - \frac{\text{Amount Recovered}}{\text{Amount Added}} \times 100.$$

$$^b \% \text{ Conjugated} = \frac{\% \text{ Disappeared} - \% \text{ Disappeared after Autoclaving}}{\% \text{ Disappeared}}.$$

It has been shown that phenolphthalein injected into rabbits is partially recovered in the urine as phenolphthalein mono- $\beta$ -glucuronide and that no sulfate esters of the phenolphthalein are found (1). Liver slices suspended in Krebs' solution free of sulfate show the same percentage disappearance and conjugation as slices incubated with sulfate. Therefore, it seems likely that no sulfate esters are formed and that probably glucuronic acid is responsible for the conjugation of phenolphthalein. The same result was obtained with the other dyes.

## DISCUSSION

Arnolt and deMeio (3) found that liver slices from one strain of rats could conjugate phenol with sulfuric or glucuronic acids. Liver slices from another strain conjugate phenol with sulfuric acid only (4). With



the latter strain, four substituted phenols are conjugated probably with glucuronic acid only, whereas stilbestrol is conjugated with both sulfuric and glucuronic acids (5). Oxidation, when it occurs, takes place only when the conjugating system is overloaded, the saturation point being specific for each compound. It is, therefore, apparent that the acid conjugated with phenols varies not only with the structure of the phenol but also with the strain of animal. It is of interest that substitution in the ring containing the phenol group has less effect on the conjugating mechanism than the substitution of a sulfonic for a carboxylic acid in another part of the molecule.

#### ACKNOWLEDGMENT

The author is deeply grateful to Dr. Frederick Bernheim for his aid and encouragement during the course of this investigation.

#### SUMMARY

1. Phenolphthalein and its tetrachloro derivative disappear when incubated with rat liver slices. The sulfonic acids, phenol red and thymol blue, disappear to a much smaller extent under the same experimental conditions.

2. Both conjugation and oxidation occur, the amount of each depending upon the concentration of the compound in question.

3. The dyes are probably conjugated with glucuronic acid.

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# Studies on Activation of Purified Alkaline Phosphatase

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Received July 28, 1947

## INTRODUCTION

The accelerating effect of magnesium on the activity of alkaline phosphatase was discovered by Erdtman (3) in 1927. In subsequent studies the use of inorganic ions in this regard has yielded results far from uniform. Jenner and Kay (4) found that, in general, the optimal concentration of magnesium for tissue phosphatase lies between 0.02 and 0.005 *M*. An excess over the optimal concentration depressed the activity. Bakwin and Bodansky (5) found about the same optimal concentration as Jenner and Kay (4) except for cattle intestine. They also found that, as the concentration of the extract was increased, the magnesium optimum increased. Armstrong (6) used a  $3 \times 10^{-4}$  *M* magnesium solution which produced an increase of about 25%. Cloëtens (7) described two types (I and II) of phosphatase in kidney. Phosphatase I is inactive in the absence of magnesium ions. Phosphatase II is active in the absence of magnesium ions. By adding magnesium to highly purified intestinal phosphatase Schmidt and Thannhauser (2) found an activation of about 25%.

Zinc ions have also been mentioned as strong activators for intestinal and kidney phosphatase (8, 9).

Amino acids are considered as important activators of alkaline phosphatase by several investigators. Bodansky (10, 11) finds that they greatly enhance the activity of bone, kidney and intestinal phosphatase. Hove *et al.* (8) use zinc salts in combination with protein split products to activate dialyzed intestinal phosphatase. Roche *et al.* (12) completely reactivate inactivated dialyzed alkaline intestinal phosphatase with glycine and different inorganic ions. Kiese and Hastings (13), Sizer (14), and Williams and Watson (15) report cysteine as a strong inhibitor of alkaline phosphatase.

Since much of the previous work on phosphatase has been based on rather crude preparations, it seemed therefore of interest to examine the behavior of purified phosphatase toward different factors influencing the activity of this enzyme—in particular, the effect of inorganic ions, amino acids and certain proteins. In some of our earlier work with alkaline phosphatase in dental tissue we had noted that the apparent yield of the enzyme was greatly increased by use of an artificial proteolysis (trypsin) for the extraction of the enzyme. A similar effect had

been noted by Ehrensvarð (1) on kidney phosphatase and by Schmidt and Thannhauser (2) on intestinal phosphatase. These observations prompted us to examine the possible effect of trypsin on purified phosphatase.

## METHODS

Phosphatase was determined by the micro method of Shinowara *et al.* (16). The inorganic phosphorus was determined according to Kuttner and Cohen (17), as modified by Kuttner and Lichtenstein (18). The substrate contained 0.5% sodium  $\beta$ -glycerophosphate (Eastman Kodak) in 0.07 *M*<sup>1</sup> sodium diethyl barbiturate. A parallel determination was always made in which 0.123 or 0.025 *M* magnesium chloride was added. The substrate buffer mixture had a pH of 9.5. The incubation was carried out at 37°C. for 15 minutes. Blanks were run with every experiment.

*Enzyme Preparations.* Samples of intestinal phosphatase purified according to Schmidt and Thannhauser were used in all experiments. Samples of purified phosphatase from calf intestines were kindly supplied to us by Dr. Gerhard Schmidt. Trypsin Difco was used in concentrations of 0.2 or 0.4 g./l. Trypsin samples from Coleman and Bell Co. and Eimer and Amend gave essentially the same results. The inorganic ions present in the trypsin preparations were Ca, Mg and Fe. There was no detectable zinc. Determination of inorganic phosphorus in the trypsin solution gave 1.3  $\gamma$ /ml. of a 0.02% solution. Incubation of phosphatase with trypsin without substrate showed no increase of the inorganic phosphorus.

Experiments were also carried out with crystalline trypsin and crystalline bovine serum albumin.<sup>2</sup>

The experiments with activating substances were carried out as follows: 0.1 cc. of the phosphatase solution was diluted to 10 cc. with water. To 2 cc. of this solution 4 cc. of the solution of the activators were added, together with a few drops of chloroform. The pH was adjusted to the desired value by means of sodium diethyl barbiturate solution in amount to yield a final concentration of 0.02 *M*. The solutions were left in glass-stoppered tubes at room temperature. At various intervals, the phosphatase activity was determined in suitable aliquots of the standing mixtures. We should like to emphasize that in all these experiments the changes in the activity of phosphatase were brought about by the different activating substances prior to the activity determination.

## RESULTS

### *Magnesium*

The data in Table I show the changes of the phosphatase activity which occur in samples of this enzyme after dilution and upon standing

<sup>1</sup> Shinowara *et al.* (16) used 0.02 *M* sodium diethyl barbiturate; others have used 0.1 *M*. Our preference for the 0.07 *M* solution is based on a comparison of phosphatase values using these three molarities.

<sup>2</sup> Crystalline trypsin was kindly supplied by Dr. M. A. Kunitz of the Rockefeller Institute at Princeton, N. J.; the crystalline bovine serum albumin by Dr. W. L. Hughes of the department of Physical Chemistry, Harvard Medical School.

at room temperature at pH 6.5. Immediately after dilution of the phosphatase solution with water (1:100), Table I, the phosphatase activity as measured by the liberation of phosphorus during a hydrolysis for 15 minutes was 0.835 mg. phosphorus and the activating effect of the Mg ions was only 10%. After 24 hours standing, the activity in the same sample fell to 0.572 mg. phosphorus and the activating effect

TABLE I

*Activation of Phosphatase by Magnesium Ions as Influenced by Time and Dilution*

Phosphatase diluted 1:100				Phosphatase diluted 1:250			
Standing at room temp.	Mg. phosphorus formed after incubation		Activation on incubation with $MgCl_2$	Standing at room temp.	Mg. phosphorus formed after incubation		Activation on incubation
Time days	No $MgCl_2$ in substrate	0.025 $M$ $MgCl_2$ in substrate	Per cent	Time days	No $MgCl_2$ in substrate	0.025 $M$ $MgCl_2$ in substrate	Per cent
0	0.835	0.918	10	0	0.626	1.060	69
1	0.572	0.920	60				
7	0.205	0.435	112	7	0.146	0.440	200
14	0.084	0.241	185				
28	0.057	0.176	210				

of Mg ions increased to 60%. When the period of standing was further extended, the activity fell to 0.057 mg. phosphorus and the activating effect was increased to 210% after 4 weeks. The difference in the activating effect of the Mg ions is not solely dependent upon the time of standing (left side of table) but also upon the dilution of the phosphatase (right side of table). At dilution 1:250 Mg caused a 69% activation at zero hour; with 1:100 dilution, activation of this degree (60%) was obtained only after standing for 24 hours. It is significant to note here also that the sample of phosphatase on which the data in Figs. 6, 7 and 8 are based had been standing in the refrigerator in concentrated form during many months (eight) and had lost a large part of its original activity according to information supplied by Dr. Schmidt.

With this sample the activation produced by adding Mg ions to the substrate was unusually high (500% or more). In another sample of phosphatase, having an original activity of about  $\frac{1}{2}$  that used in the preceding experiment, the activation by Mg ions when added to the substrate after 1:50 dilution and immediate incubation was 30%.

When the same phosphatase was standing in the ice box for 5 days, the activating effect of the Mg ions rose to 36.5% when diluted 1:50 and immediately incubated. After an additional 3 days standing the activating effect rose to 54%. From these experiments it was obvious that alkaline intestinal phosphatase on standing and dilution (aging) loses its activity which can be regained completely or in part by adding an optimal concentration of Mg ions to the substrate during activity determination.

It became of interest, therefore, to examine the behavior of phosphatase when diluted and let stand at room temperature in the presence of Mg ions in the concentration used in the substrate during activity determination, *i.e.*, 0.025 *M* MgCl<sub>2</sub>. Such an experiment is shown in Table II. Here phosphatase was diluted 1:50 and buffered

TABLE II  
*Phosphatase Standing with Magnesium*

Standing at room temp.			Mg. phosphorus formed after incubation			Activation on incubation with 0.025 <i>M</i> MgCl <sub>2</sub>	Activity on incubation with $4.16 \times 10^{-4}$ <i>M</i> MgCl <sub>2</sub> as compared with the activity produced on incubation with 0.025 <i>M</i>
Days	pH	MgCl <sub>2</sub> added	No MgCl <sub>2</sub> in substrate	0.025 <i>M</i> MgCl <sub>2</sub> in substrate	$4.16 \times 10^{-4}$ <i>M</i> MgCl <sub>2</sub> in substrate	Per cent	Per cent
0	6.5	none	0.475	0.735	0.620	54.6	84.4
0	6.5	0.025 <i>M</i>	0.620	0.735			
1	6.5	none	0.386	0.636	0.510	65.0	80.3
1	6.5	0.025 <i>M</i>	0.478	0.464			
4	6.5	none	0.205	0.352	0.274	71.8	77.7
4	6.5	0.025 <i>M</i>	0.133	0.136			
0	9.5	none	0.450	0.720	0.608	60.0	84.5
0	9.5	0.025 <i>M</i>	0.620	0.724			
1	9.5	none	0.283	0.376	0.328	33.0	87.2
1	9.5	0.025 <i>M</i>	0.308	0.308			
4	9.5	none	0.133	0.191	0.151	43.5	79.0
4	9.5	0.025 <i>M</i>	0.137	0.137			

to pH 6.5 or 9.5. The significant points to be noted are: (1) phosphatase lost its activity more rapidly when Mg ions were present in the standing solution than when they were absent, regardless of pH; (2) after the phosphatase has been standing with Mg the activating effect of adding Mg ions to the substrate in optimal concentration was entirely lost;

(3) phosphatase lost its activity more rapidly when standing at pH 9.5 than at pH 6.5, irrespective of whether or not Mg ions were present in the standing solution; and (4) with no Mg added to the standing solution the activation produced by adding an optimal concentration of this ion to the substrate steadily increased with the duration of standing at pH 6.5 (similar to Table I), but at pH 9.5 there was an initial decline in this activating effect after 24 hours standing. However, using a suboptimal quantity of Mg ion ( $4.16 \times 10^{-4} M$ )<sup>3</sup> in the substrate there was a steady decrease in the activating effect at pH 6.5 as compared with that produced by the optimal concentration (0.025 *M*). At pH 9.5 it will again be noted that there is an exceptional finding after 24 hours standing—there being an increase in the per cent activation on the same basis of comparison.

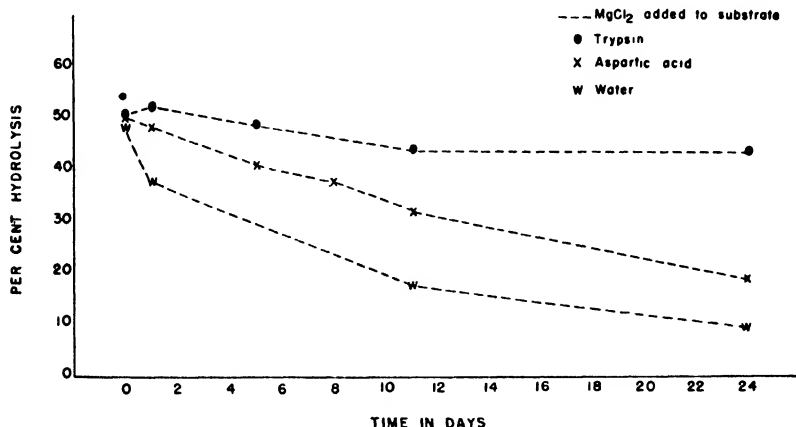


FIG. 1. Influence of trypsin and of aspartic acid on phosphatase activity when standing at room temperature and pH 9.5 for 24 days. Concentration of trypsin solution—0.02%. Concentration of aspartic acid— $1 \times 10^3 M$ . 0.025 *M*  $MgCl_2$  in substrate where indicated.

### Amino Acids

Since it has been demonstrated by Bodansky and others that amino acids have an enhancing effect on the activity of alkaline phosphatase,

<sup>3</sup> This is the concentration of  $MgCl_2$  found in the substrate in samples which had been standing with 0.025 *M*  $MgCl_2$  and which, had been subsequently diluted for the activity determination. The data obtained at this concentration serve as a control for the samples of phosphatase which had been standing with  $MgCl_2$  and then incubated without further addition of  $MgCl_2$ .

we investigated the effect of several amino acids, namely, alanine, aspartic acid (asparagine), glycine, lysine, tyrosine and tryptophan, on purified alkaline intestinal phosphatase and found that aspartic acid had the strongest and most lasting activating effect.

A sample of highly active (fresh) phosphatase standing with aspartic acid buffered at pH 9.5 at room temperature for a period of 24 days was compared with a sample of phosphatase standing with water buffered to the same pH (Fig. 1). The decrease of the phosphatase activity with water was 80%, that after standing with aspartic acid, 61%. When inorganic ions were added to aspartic acid, it was observed that the

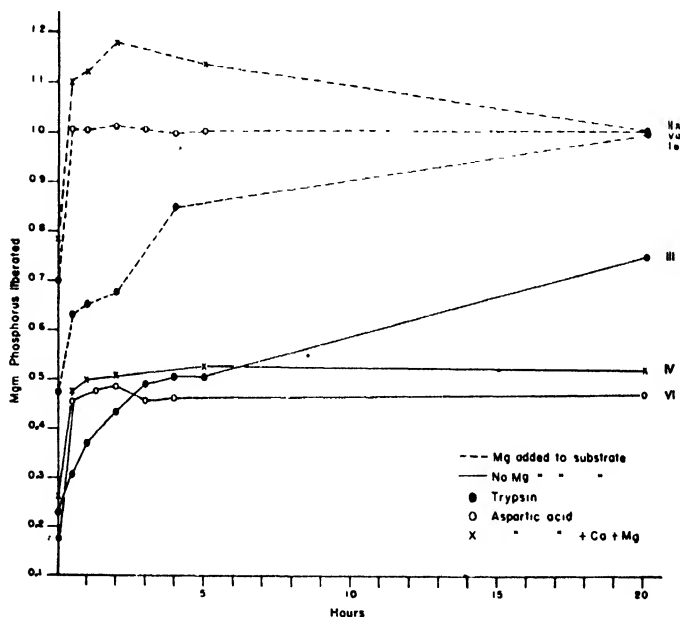


Fig. 2. A comparison of the activating effects of trypsin, aspartic acid and a mixture of aspartic acid plus inorganic ions. All samples were buffered at pH 9.5. Concentration of trypsin solution—0.02%. Concentration of aspartic acid— $1 \times 10^{-3}$  M. Inorganic ions, Ca and Mg, in concentration of  $1 \times 10^{-3}$  M;  $1.9 \times 10^{-3}$  M, respectively. 0.025 M  $\text{MgCl}_2$  in substrate where indicated.

activating effect of a mixture of an amino acid and of the salts was higher than that of the amino acid alone (Fig. 2). Similar effects were obtained by Hove *et al.* (8) and Roche *et al.* (19), who found that the activating effect of either of the two substances (amino acid and inorganic ions) was smaller when used separately than in combination.

On investigating in more detail the action of amino acids on phosphatase within the first 24 hours, we found that the maximal activating effect of amino acids was always reached in a rather short time. This could be demonstrated however only when Mg ions were added to the substrate during the activity determination. Under such conditions, the

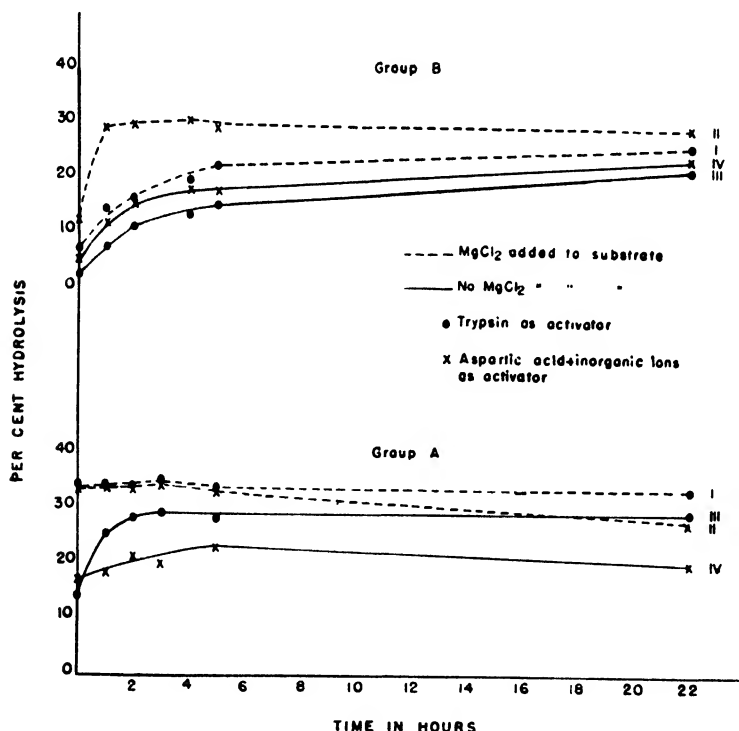


FIG. 3. Influence of age of phosphatase solution on the response to activators. Group A, phosphatase used immediately after dilution; Group B, after 2 weeks. Concentration of the trypsin solution—0.02%. Concentration of aspartic acid— $1 \times 10^{-2}$  M. Inorganic ions Ca, Mg, Fe, in concentrations of  $1 \times 10^{-3}$  M,  $1.9 \times 10^{-6}$  M and  $1 \times 10^{-5}$  M, respectively.

time required to reach maximal activity was not influenced by aging of the phosphatase. It should be noted that, without the addition of magnesium to the substrate, the activity gradually increases during 24 hours in samples of aged phosphatase.

The effect of a mixture of amino acid plus inorganic ions on phosphatase samples of different age is illustrated in Figs. 3 and 4. Note



that in Group A (Figs. 3 and 4) the activator (a mixture of amino acid plus inorganic ions) was added to the phosphatase immediately after dilution, and in Group B, the same phosphatase had been standing 8–14 days at room temperature prior to the addition of the same activator. The maximal activity was reached within 3–4 hours in both

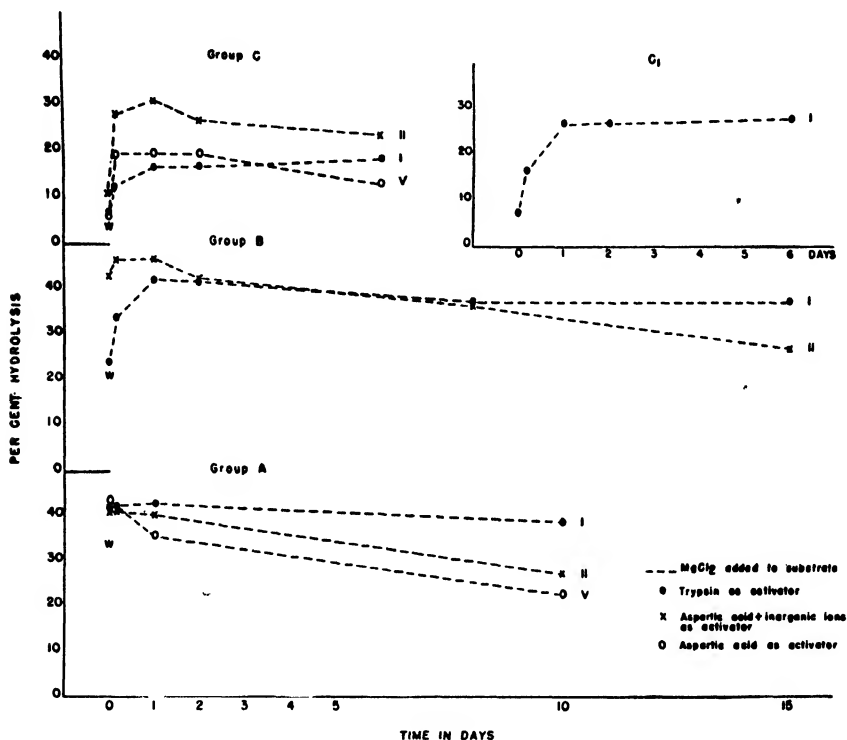


FIG. 4. Influence of the age of phosphatase solution on the response to activators. Group A, phosphatase used immediately after dilution; Group B, after 8 days; Group C, after 16 days; C<sub>1</sub> phosphatase sample same as for Group C, but standing for 24 hours in alkaline medium prior to the addition of trypsin. W represents the original activity of phosphatase. 0.025 *M* MgCl<sub>2</sub> in the substrate.

instances. A much longer period of time was required for maximal activity to be reached when polypeptides, as present in trypsin solutions (Fig. 3), were used to activate phosphatase as described in the following section. This difference is illustrated further in Figs. 2, 3 and 4.

Fig. 5 illustrates the influence of pH on the activation produced in phosphatase samples of different initial activity by a mixture of aspartic acid plus inorganic ions as measured after 24 hours; in Group A the enzyme was highly active, in Group B the starting activity was very low. The dependency of the activating action on the pH of the standing solution is quite obvious in each instance.

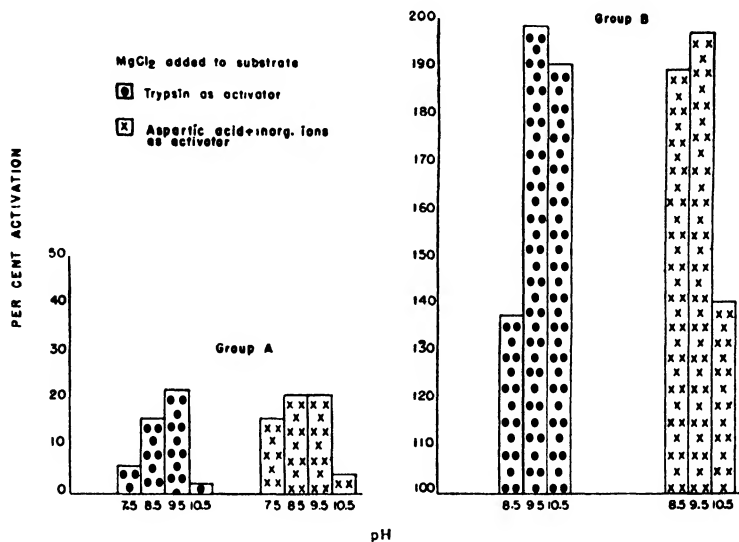


FIG. 5. Influence of pH on the activation of phosphatase solutions of different initial activity. Activity determinations were made at zero and 24 hours after the activators were added and the pH adjusted to the indicated values with sodium diethyl barbiturate. All substrates were adjusted to yield a final pH of 9.5 for the activity determination. Concentration of trypsin 0.02%; aspartic acid,  $1 \times 10^{-3} M$ ; inorganic ions Ca,  $1 \times 10^{-5} M$ ; Mg,  $1.9 \times 10^{-6} M$ ; and Fe,  $1 \times 10^{-5} M$ .  $MgCl_2$ , 0.025  $M$  in the substrate.

### *Trypsin*

When commercial trypsin was allowed to stand with aged samples of phosphatase which had lost most of their original activity, we found that there was a gradual recovery of activity which was sustained for a remarkably long time. The question to be answered first was whether this reaction was due to a direct enzymatic effect on the phosphatase or to the presence of non-enzymatic activator in the trypsin samples.

On the basis of the following data we concluded that at least a large

part of the activation of phosphatase by trypsin is attributable to the presence or formation of activating non-enzymatic substances:

(1) *Ashed trypsin* showed no activating influence on the activity of phosphatase.

(2) *The inorganic ions Ca, Mg and Fe*, on standing with phosphatase, in concentrations corresponding to the composition of the trypsin solution, had no activating effect regardless of pH.

(3) *Dialyzed trypsin*. Dialyzed trypsin and the dialyzate each produced an increase in phosphatase activity. Both effects were weaker than that of the original trypsin solution. Fig. 6 represents a summary

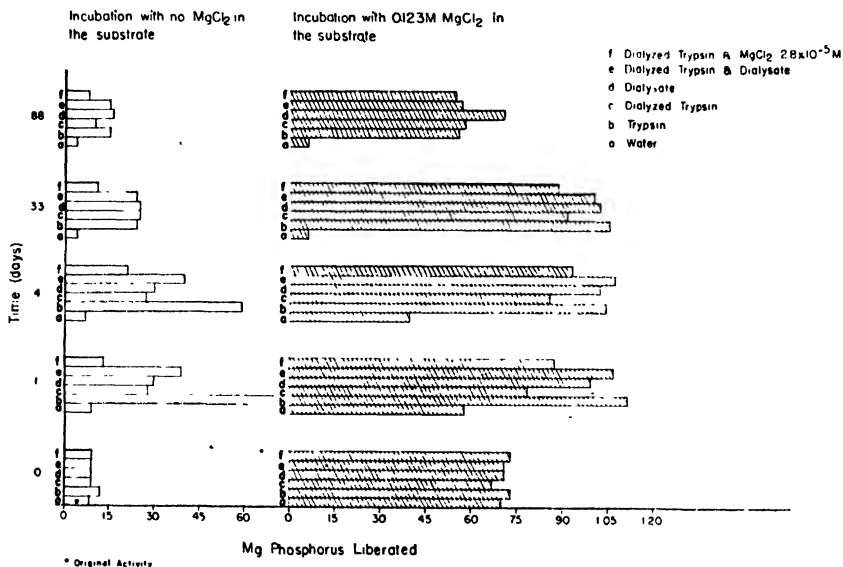


Fig. 6. Influence of commercial trypsin and dialyzed commercial trypsin and its dialyzate on the activity of phosphatase. All solution were adjusted to pH 9.4 with sodium diethyl barbiturate.

of an experiment with dialyzed trypsin solution. It is pertinent to note that the two samples of the dialyzed trypsin (columns c and f) produced on incubation with Mg a gradual rise in phosphatase activity during 4 days, whereas all other samples so incubated show a maximal activity within 24 hours.

(4) *Inactivated trypsin*. Heating the trypsin on a steam bath for 2 hours at pH 9.5 did not destroy its activating effect, but this was slightly less and required a longer time.

(5) *Inactivated and dialyzed trypsin.* Since the action of trypsin was mainly due to products of proteolysis, these products being already present or formed anew when the dialysis of non-inactivated trypsin was stopped, we investigated the effect of inactivated dialyzed trypsin. The data in Table III are based on two samples of phosphatase of

TABLE III  
*Effect of Inactivated Dialyzed Trypsin on Phosphatase Activity*

Group A				Group B			
Standing at room temp.	Mg. phosphorus liberated after incubation. 0.025 M MgCl <sub>2</sub> in the substrate			Standing at room temp.	Mg. phosphorus liberated after incubation. 0.025 M MgCl <sub>2</sub> in the substrate		
Time hours	Trypsin	Inactivated trypsin dialyzed	Alkaline trypsin dialyzed	Time hours	Trypsin	Inactivated trypsin dialyzed	Alkaline trypsin dialyzed
0	0.607	0.570	0.621	0	0.343	0.181	0.208
3	0.850	0.719	0.740	24	0.800	0.415	0.486
24	1.058	0.869	0.991	48	0.807	0.434	0.523
48	1.045	0.892	0.892	145	0.740	0.290	0.575
192	0.956	0.818	0.898				
375	0.946	0.763	0.898				

different initial activity which were tested with (1) inactivated dialyzed trypsin and (2) alkaline dialyzed trypsin. It can be seen that the maximal action of each dialyzed solution was less than the action of the intact trypsin. With the intact trypsin and the alkaline dialyzed trypsin solution, there was still the specific stabilizing effect present, whereas with the inactivated dialyzed sample this effect was considerably diminished.

The following experiments illustrate the activating effect of the non-enzymatic protein split-product (peptone or polypeptide present or formed in commercial trypsin solutions) on purified alkaline phosphatase.

Fig. 7 shows the effect of crude trypsin on alkaline phosphatase when buffered at pH 9.3. After various time intervals during incubation with trypsin at room temperature, determinations of phosphatase activity with and without the addition of magnesium chloride to the substrate, were carried out. In the lower curve (phosphatase standing with water buffered at pH 9.4) the activity fell off after a brief early rise. It can be

seen that the sample with magnesium chloride in the substrate has its maximal activity at zero hour. On standing, the phosphatase lost activity and the response to magnesium fell off even more rapidly. In the samples standing with trypsin solution there was not only a marked

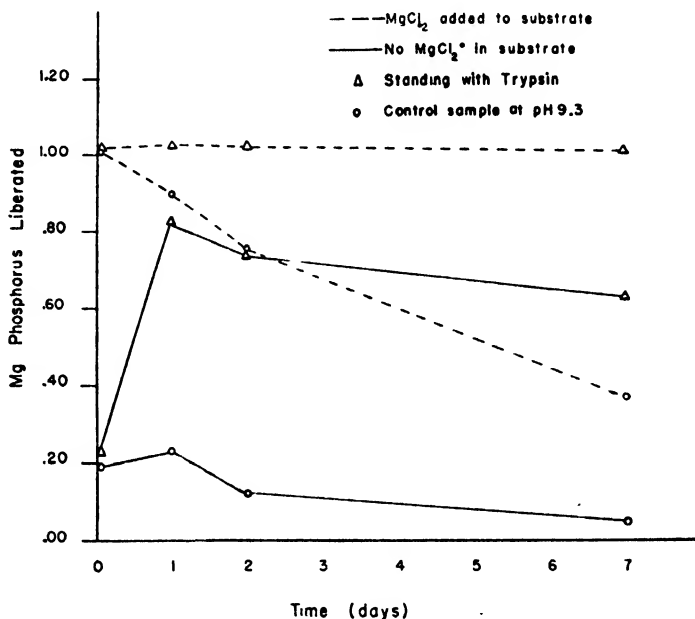


FIG. 7. Influence of trypsin (commercial) on phosphatase activity. Phosphatase standing at room temperature with 0.02% solution of trypsin and with water during 7 days. Both solutions buffered at pH 9.3. 0.125 *M* MgCl<sub>2</sub> in substrate where indicated.

increase in the phosphatase activity during the first 24 hours but the activity in the presence of magnesium was retained at a high level throughout the 7 days of the experiment.

A similar experiment of much longer duration is shown in Fig. 8. It should be noted that the initial phosphatase activity at zero hour was unusually low. A short rise in phosphatase activity was observed in the sample standing with water when incubated without magnesium. After 33 days the difference in the capacity of phosphatase to be activated by magnesium after standing with trypsin as against water becomes extreme; with trypsin this reaction is unimpaired, without trypsin it is almost completely lost. Although the activity of phos-

phatase standing with buffered water diminishes greatly, we could always detect a residual activity, even after standing at room temperature for many months. However, this activity was markedly or completely depressed when various amounts of Mg ions were added to the

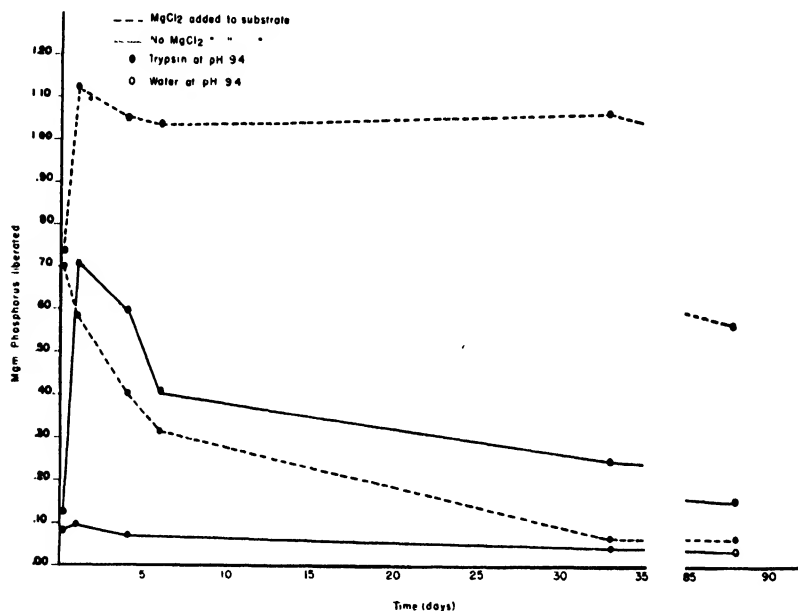


FIG. 8. Influence of trypsin (commercial) on phosphatase activity. Phosphatase standing at room temperature with 0.02% trypsin solution and with water during 88 days. Both solutions buffered at pH 9.4.

substrate during incubation. Jenner and Kay (4) also observed a small residue of phosphatase activity in animal tissue extracts which could not be eliminated by dialysis or electrodialysis.

A more detailed study of the action of trypsin on phosphatase within the first 24 hours showed that the activating effect was a time reaction, the nature of which depends on several factors relating to phosphatase, such as aging and pH. The effect of aging is illustrated in Fig. 9. A highly active phosphatase sample treated with trypsin immediately after dilution yielded maximal activation after 4–6 hours (Group A) regardless of whether the determination of phosphatase activity was carried out with or without Mg in the substrate. When the same phosphatase sample was left at room temperature for 8 days prior to the addition of trypsin (Group B), the maximal activity was reached only after 24 hours. We found that the pH also influences the action of trypsin on phosphatase (Fig. 10). At 24 hours the activity was maximal at

pH 9.5 and the activation produced by Mg ions when added to the substrate decreased with increasing pH (in the standing mixture). At 7.5 the activation was 90%, at 8.5, 34%, at 9.5, 21.5% and at 10.5, 11.5%. After 7 days standing and with Mg added to the substrate the phosphatase activity dropped slightly from the 24 hour level at pH 9.5, showed a slow rise at 7.5 and 8.5, but a marked decline at 10.5. On incubation

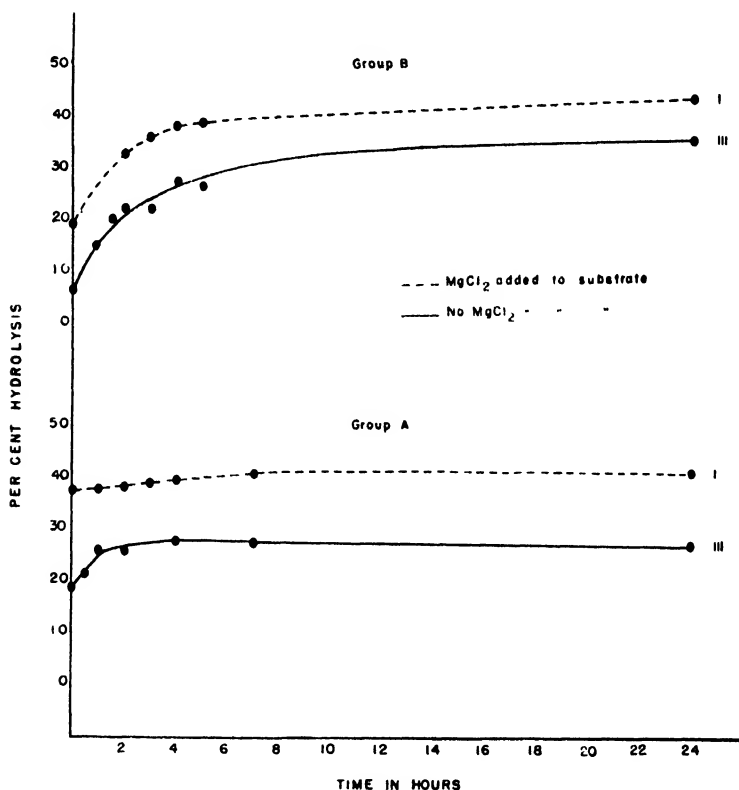


Fig. 9. Influence of age of phosphatase on its activation by trypsin. Group A, fresh sample of phosphatase standing with trypsin at pH 9.5; Group B, sample of phosphatase diluted and then let stand 1 week at room temperature prior to adding trypsin and adjusting pH to 9.5. 0.025 *M* MgCl<sub>2</sub> in substrate where indicated.

without Mg there was a slight decline at all pH levels except 7.5 which showed an increase. As a control for this experiment we used samples of phosphatase standing with (1) water buffered at these same pH values, and (2) with Ca, Mg and Fe salts corresponding to the concentration of the trypsin solution and also adjusted to these same pH values. The results show a decline in activity in all samples standing 24 hours without trypsin.

It was observed that the activating power of the trypsin solution decreases with the age of the phosphatase. In Group A (Figs. 3 and 4) the phosphatase was used immediately after dilution. The maximal

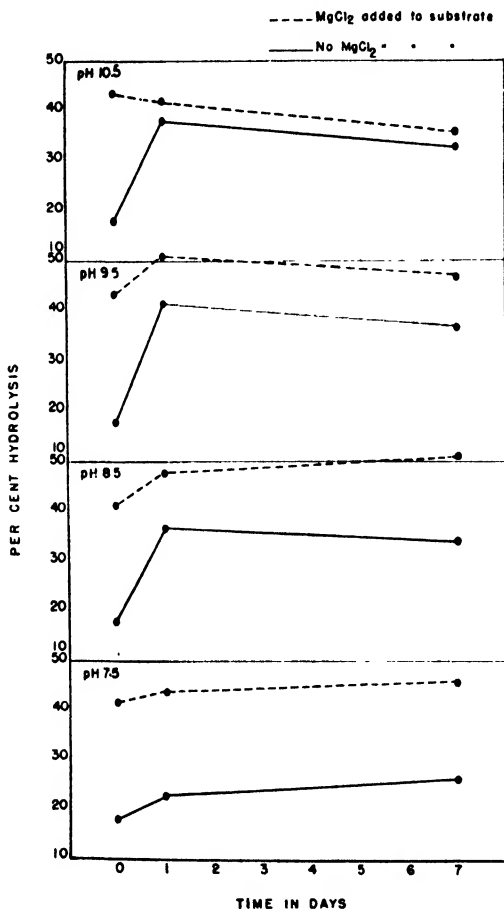


FIG. 10. Influence of pH on the activation of phosphatase by trypsin. The pH of the solutions was adjusted with sodium diethyl barbiturate according to Michaelis. All substrates were adjusted to yield final pH of 9.5 for the activity determination. 0.025 *M* MgCl<sub>2</sub> in substrate where indicated.

activity was reached within 3 hours. In Group B the phosphatase was diluted with distilled water and left at room temperature 8 days before trypsin was added. The activity rose slowly during 24 hours and



reached 89% of the activity obtained in a comparable sample standing with aspartic acid. In Group C the phosphatase was left still longer (16 days) prior to the addition of activators. In the trypsin sample the phosphatase activity rose slowly throughout the duration of the experiment (6 days) and reached only 57% of the activity observed in a like sample standing with aspartic acid plus Mg ions. We tested, in addition, a more concentrated solution of trypsin. The activity was the same as that reached with the weaker trypsin solution.

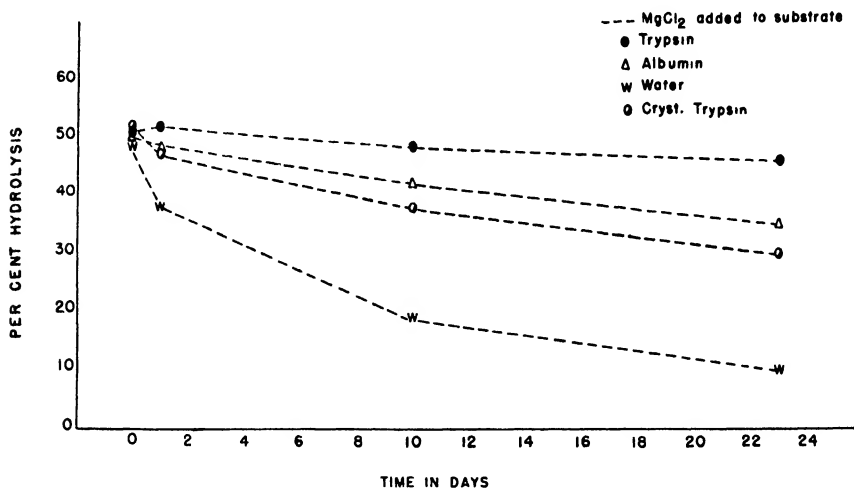


Fig. 11. Influence of crystalline serum albumin and crystalline trypsin on phosphatase activity. Concentration of trypsin—0.02%; concentration of albumin, 17.8 mg. protein/100 ml.; concentration of crystalline trypsin 22.5 mg. protein/100 ml. All solutions were adjusted to pH 9.4 with sodium diethyl barbiturate.

Further evidence for the dependency of the trypsin action on the pH was obtained in the experiment represented in Fig. 4, Group C<sub>1</sub>. Here, after 16 days in distilled water, the phosphatase was made alkaline and let stand for an additional 24 hours prior to adding trypsin. The action of trypsin, although still a time reaction, was greatly accelerated and the maximal activity reached the level produced by aspartic acid plus inorganic ions (compare with Group C).

Crystalline trypsin and crystalline bovine serum albumin both have a similar activating action on phosphatase activity (Fig. 11). Both activate phosphatase but do not exhibit the characteristic stabilizing effect of crude trypsin.

## DISCUSSION

The activity of alkaline phosphatase decreases very markedly during aging when diluted with distilled water buffered at pH 6.5 and 9.5 and let stand at room temperature. In fresh samples of phosphatase the activating effect produced by adding Mg ions to the substrate is relatively small and increases steadily during the aging period (Table I). These findings seem to indicate that the phosphatase molecule itself contains a certain amount of inorganic ions sufficient for complete activation.

The inactivation of phosphatase while standing at room temperature at pH 6.5 or 9.5 was accelerated by increasing the dilution and by the addition of Mg ions to the standing solution (Tables I and II). At pH 6.5 the percentage activation produced by adding Mg to the substrate increased steadily during aging in the absence of Mg. At pH 9.5 using the same procedure the percentage activation dropped from 60 to 33; the temporary aspect of this change was shown by the fact that at 4 days a value of 43.5% was obtained. This suggests that, under these conditions, the phosphatase, especially its inorganic group, undergoes changes which can partly and *temporarily* be reversed by standing at alkaline pH. The regeneration (at alkaline reaction), makes it possible for this group to participate in the activation of phosphatase. This phenomenon is manifest on incubation with zero or suboptimal concentration of  $\text{MgCl}_2$  in the substrate ( $4.16 \times 10^{-1} M$ ).

Wood and Ross (22) found that proteins, such as egg albumin and serum albumin, increase the liberation of phosphate by kidney phosphatase, but came to the conclusion that the activation by these substances was somewhat different from that caused by amino acids, ammonia, and veronal. We, likewise, found that crystalline bovine serum albumin and crystalline trypsin activate purified intestinal phosphatase but their action cannot be classed with that produced by commercial trypsin, in that they did not preserve the phosphatase activity nearly so well. Since both the dialyzate of commercial trypsin and the dialyzed trypsin were found to have a similar, though somewhat smaller, action than that of intact trypsin, the assumption was made that a protein-split product present in the trypsin solution, or formed on standing, was the cause of the typical activating and stabilizing action of trypsin. Moreover, when trypsin was inactivated and then dialyzed, we found that the stabilizing effect was greatly decreased in

comparison with that of a dialyzed alkaline trypsin solution which had not been previously inactivated. We interpreted these findings as follows. The activating effect of dialyzed trypsin was due in part to the trypsin protein but the stabilizing effect was attributed to a polypeptide formed after the dialysis was stopped (see Fig. 10, columns c and f). In the inactivated dialyzed trypsin solution the activating effect was primarily due to the trypsin protein; here no new polypeptide could be formed after dialysis and the specific stabilizing effect was now lost or greatly diminished (Table III).

That the nature of the activation produced by this "polypeptide" is different from that produced by amino acids is concluded from the following data. The action of amino acids is, in most instances, a rather rapid one, and the rate is independent of the age of the phosphatase solution. Moreover, once the maximum activation is reached, a steady decline is always observed. The rate of trypsin (polypeptide) action on the other hand is dependent upon the state of phosphatase present. When the phosphatase is highly active, the trypsin action is relatively rapid, whereas the less the activity of the phosphatase (as brought about by aging), the more time is required for it to reach maximal activity. Not only is this time prolonged, but also the maximal values reached here are lower than those obtained with the amino acids. Once this maximal activation of phosphatase by trypsin is reached, it persists for a long time. The existence of some activator besides amino acids has already been anticipated by Hove *et al.* (8) who came to the following conclusion in their investigations on dialyzed intestinal phosphatase: "It has not been established that the amino acids liberated by the autolysis of the intestinal mucosa are the only compounds with a zinc-coactivating property present in the crude preparation."

When trypsin was added to aged samples of phosphatase and the solution buffered at pH 9.5, the activity of phosphatase was strikingly enhanced. This is a time reaction which is influenced by the pH of the standing mixture (Fig. 10) and by the aging of phosphatase (Fig. 3). These results suggest the following explanation. At pH 9.5 the regeneration of an inorganic group, as pointed out above, makes it possible for this group to participate in the activation of phosphatase and when trypsin is present, other activating groups are supplied. At a higher pH (10.5) the inorganic ions participate to a higher degree in the activation of the enzyme, but, on the other hand, the phosphatase is destroyed more rapidly. At pH 8.5 and 7.5 the activity is well preserved

but the regeneration of the inorganic group is much slower; consequently, with decreasing pH of the standing trypsin-phosphatase solution the addition of a constant amount of Mg to the substrate is able to produce an increasingly greater activation. The described effect of trypsin on phosphatase at alkaline pH suggests that the activating influence of the polypeptide present in the trypsin solution involves only the active phosphatase metal protein, and not the inactive protein formed from phosphatase during aging. This interpretation is strongly supported by the experiment in which phosphatase was first incubated a short time in alkaline medium before trypsin was added (Fig. 8, group C<sub>1</sub>). Here the action of trypsin was greatly speeded up and the activity in such samples reached the level of the activation obtained with amino acid.

Since some of the activating effects on phosphatase described in this paper represent time reactions, it is of interest that similar examples of activation have been reported in the literature for other enzymes. Smith and Bergmann (20, 21) found, in 1941, that the activation of intestinal peptidase by Mg requires about 3 hours and, in the case of leucine aminoxopeptidase, a still longer time is required for maximal activation. The rate of reaction and the maximal activity are dependent upon the concentration of the metal.

These experiments on purified alkaline phosphatase enabled us to develop a method for the histochemical demonstration of this enzyme in decalcified specimens of bones and teeth. By decalcification of the tissues from 28-day old rats at pH 4.8–5.0, followed by dehydration in alcohol solutions buffered to pH 9.4, and by incubating the sections in aqueous sodium diethyl barbiturate buffer of pH 9.4 prior to the actual test for phosphatase, we were able to preserve the activity of tissue phosphatase to a considerable extent (Greep, Fischer and Morse, 23, 24).

#### ACKNOWLEDGMENT

We gratefully acknowledge our indebtedness to Dr. Gerhard Schmidt for generously supplying us with his purified alkaline phosphatase and also for reading this manuscript.

#### SUMMARY AND CONCLUSIONS

1. Purified alkaline phosphatase loses its activity during standing at room temperature at pH 6.5 and 9.5.
2. Phosphatase activity can be partly or completely restored when an optimal concentration of Mg ions is added to the substrate.

3. On standing with Mg, phosphatase loses its activity more rapidly and can no longer be activated by adding Mg to the substrate.

4. The striking difference between the relatively small activating effect produced by adding Mg to the substrate during the incubation of fresh (highly active) phosphatase, as compared to the strong effect with aged samples having reduced activity, can be explained by the assumption that highly active phosphatase itself contains a sufficient amount of metal for its maximal activation.

5. In aged samples of phosphatase the activity can be restored by allowing the phosphatase to stand with amino acids or with a commercial trypsin at room temperature.

6. From the results of experiments with dialyzed and with ashed trypsin it is concluded that the activating and stabilizing effect of commercial trypsin is due to the presence of a polypeptide.

7. The effect of trypsin and amino acids is a time reaction. The time period required for the maximal trypsin effect is dependent on changes in the phosphatase which occur on previous aging whereas that of amino acids is independent of these changes.

8. The maximal activation produced by the commercial trypsin solution persists for a long time. The activation produced by amino acids reaches a maximum and then declines.

9. The observations described in this paper suggest that phosphatase is a metal protein and that the inactivating and reactivating effects involve the specific nature of the metal group of the enzyme protein.

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# Studies on Lactoglobulins <sup>1</sup>

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Received August 1, 1947

## INTRODUCTION

In the course of a study of milk proteins (1), it was noted that goat milk whey contained a protein which was relatively sharply defined electrophoretically and which constituted about 60% of the total whey proteins. Since the alcohol fractionation methods of Cohn *et al.* (2) have not been extensively applied to protein systems other than blood plasma, it appeared desirable to study their application to the milk whey protein system. The immediate objective of the fractionation procedure was the isolation, in electrophoretically pure form, of the above mentioned protein to which we will refer hereafter as "goat lactoglobulin."

The report of Li (3) on the presence of more than one electrophoretic component in crystalline bovine  $\beta$ -lactoglobulin stimulated us to work out the conditions for its preparation by ethanol fractionation and to compare its electrophoretic behavior at various pH's with the somewhat analogous lactoglobulin from goat milk.

A description of the fractionation procedures and certain of the physical properties of the above lactoglobulins form the subject of this paper.

## EXPERIMENTAL

Fractionation of both bovine and goat lactoglobulins was carried out by means of the alcohol precipitation methods introduced by Cohn *et al.* (2) which have been hitherto used chiefly to separate certain of the components of blood plasmas (2, 4, 5,

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<sup>1</sup> This work was supported by grants from the Rockefeller Foundation and the Wisconsin Alumni Research Foundation.

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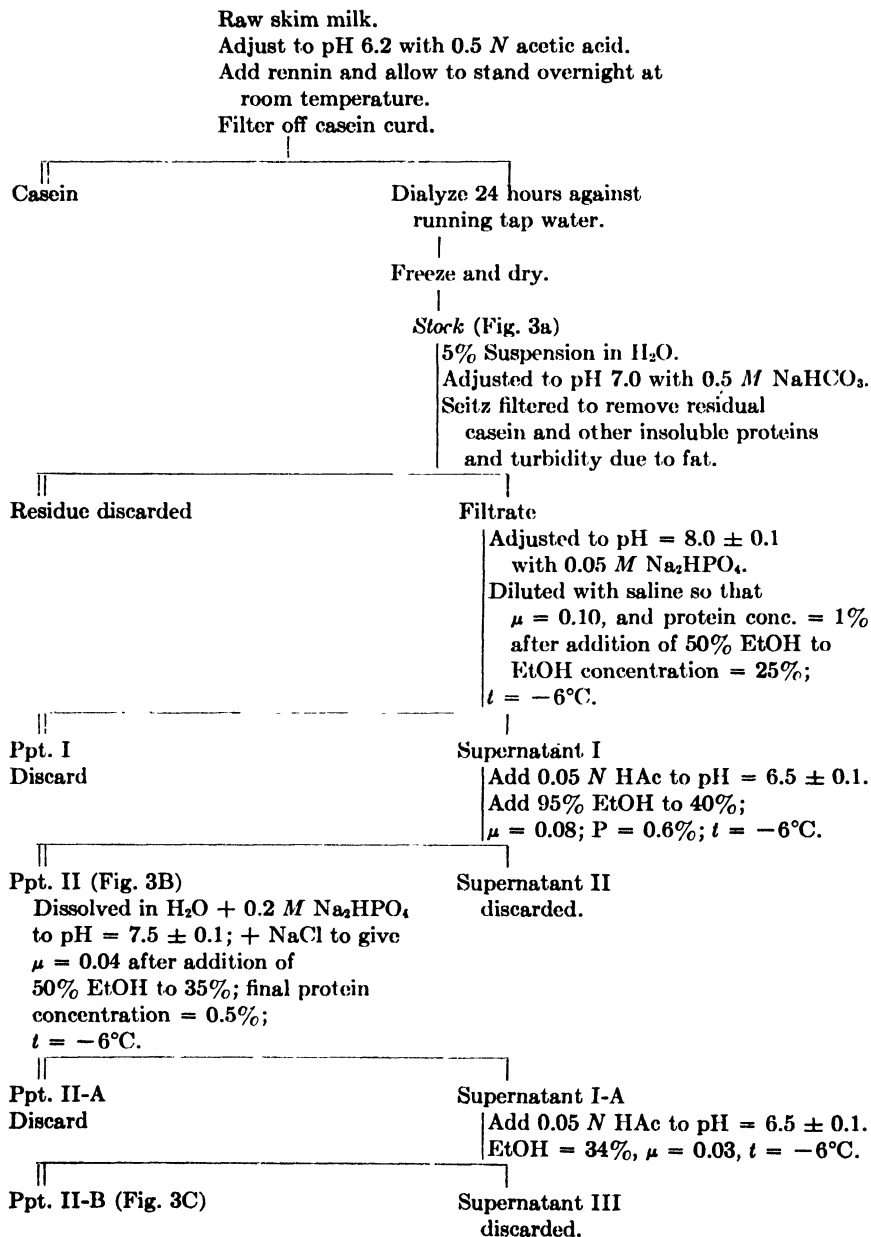


FIG. 1. Fractionation scheme for goat lactoglobulin.

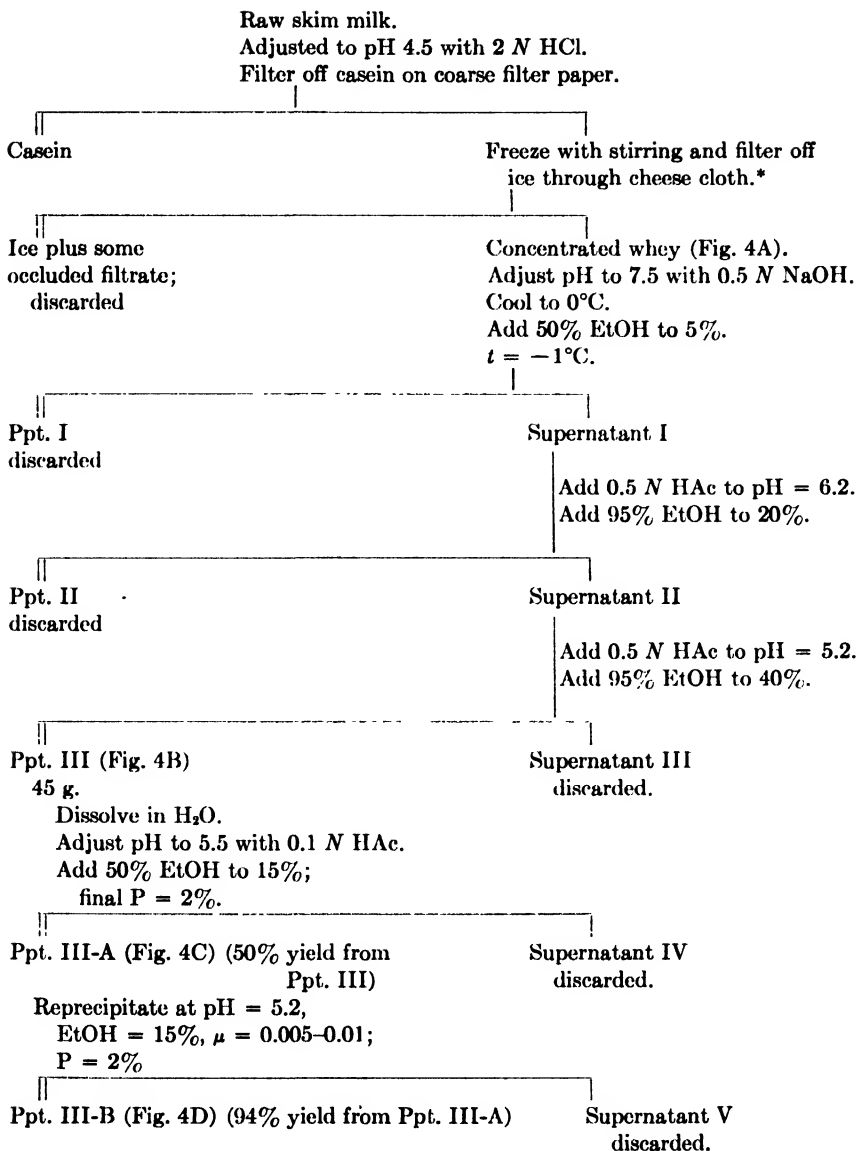


FIG. 2. Fractionation scheme for bovine lactoglobulin.

\* An alternative method of concentrating the whey proteins consists in freezing and drying of dialyzed whey samples as used for goat whey (Fig. 1).

6, 7, 8, 9, 10). Experiments were performed to determine the optimum conditions for the separation in good yield of the component sought. The conditions finally chosen are summarized in Figs. 1 and 2.

While the casein was separated from cow milk by isoelectric precipitation, the casein of goat milk was removed by treatment with rennin. Previous studies have shown that a lower pH is necessary to remove goat casein than is required for the bovine system. The goat wheys in which the casein is removed by isoelectric precipitation show smaller amounts of proteins of lower isoelectric point than do wheys prepared by the use of rennin (1). Since the relatively low pH required to remove casein from goat milk may tend to modify certain of the whey proteins, it appeared desirable to effect casein removal from this system by the use of rennin (Fig. 1).

Concentration of the bovine whey proteins by either drying of dialyzed samples from the frozen state, or by freezing out the major portions of the water according to the method of Palmer (11), provided material suitable for ethanol fractionation. In all cases where alcohol was present the temperature of the system was maintained at  $-6^{\circ}\text{C}.$ , or as near thereto as the freezing point of the solution would allow. The pH values were determined on 2 cc. aliquots at  $25^{\circ}\text{C}.$  Bovine  $\beta$ -lactoglobulin was also prepared by  $(\text{NH}_4)_2\text{SO}_4$  fractionation according to the method devised by Smith (9). Products were obtained which were electrophoretically the same as the ethanol-prepared proteins. However, in our hands the yields from  $(\text{NH}_4)_2\text{SO}_4$  procedures were only about half as good as those realized with ethanol.

The progress of the fractionations was followed by means of routine electrophoretic analyses carried out on 3% protein solutions in pH 8.6 barbiturate buffer of 0.1 ionic strength and at constant potential gradients of approximately 6.2 volts/cm. Mobility determinations were carried out at potential gradients of approximately 4.5 volts/cm. on 0.5% protein solutions in buffers of 0.1 ionic strength in which NaCl contributed 80% of the ionic strength, the remainder, depending on the pH, being supplied by the buffer ion; glycine, acetate, cacodylate, or barbiturate. The moving boundaries were followed in the usual Tiselius apparatus using the diagonal knife edge schlieren method. Throughout this paper only the descending electrophoretic patterns are shown, the ascending patterns being normal in every case. Sedimentation analyses were carried out on 0.5% protein solutions in 0.15 *N* NaCl in the oil-turbine ultracentrifuge at approximately 220,000 *g* using a schlieren optical system to record the boundaries.

## RESULTS

In Fig. 3 are shown the electrophoretic patterns of the products obtained at the various steps in the fractionation scheme outlined in Fig. 1 for goat lactoglobulin. A product giving a single symmetrical electrophoretic peak at pH 8.6 was obtained (Fig. 3C). The overall yield is approximately 40%, and it is possible to obtain from 10 l. of milk about 10 g. of material which is 95% pure or better under the above conditions of analysis. The same information for the ethanol separation of bovine  $\beta$ -lactoglobulin is shown in Fig. 4. Again a product

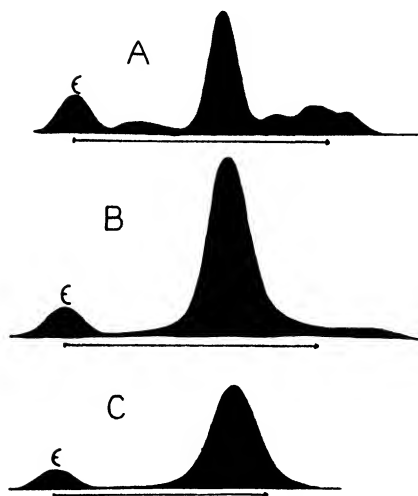


FIG. 3. Electrophoretic patterns of goat lactoglobulin fractions. Duration of experiments—7200 seconds.

was obtained which was composed, to the extent of at least 95 %, of one electrophoretic component at pH 8.6 (Fig. 4D), and whose mobility corresponds closely to that found by Pedersen (12) for this protein, namely  $5.2 \times 10^{-5}$  cm.<sup>2</sup> volt<sup>-1</sup>sec.<sup>-1</sup>. Here the yield is about 12 g. of material of the above purity/10 l. of milk. Attempts at crystallization

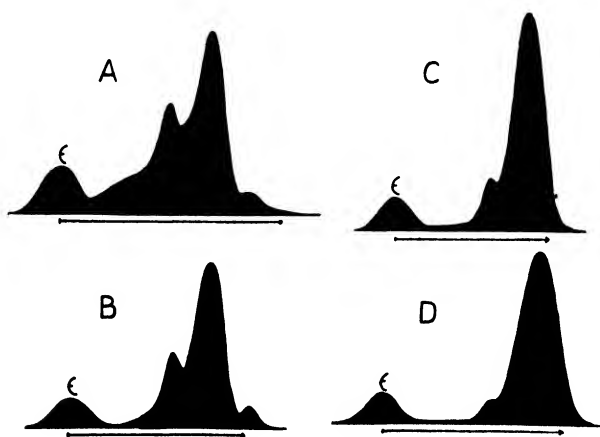


FIG. 4. Electrophoretic patterns of bovine lactoglobulin fractions. Duration of experiments—7200 seconds.

of both of these products prepared by ethanol fractionation have not yet met with success.

When the goat lactoglobulin was studied electrophoretically over the pH range 3.0–10.0, it was found that it split into two components, and at one pH, into three. The details of this electrophoretic splitting may be followed diagrammatically in Fig. 5A. It will be seen that only

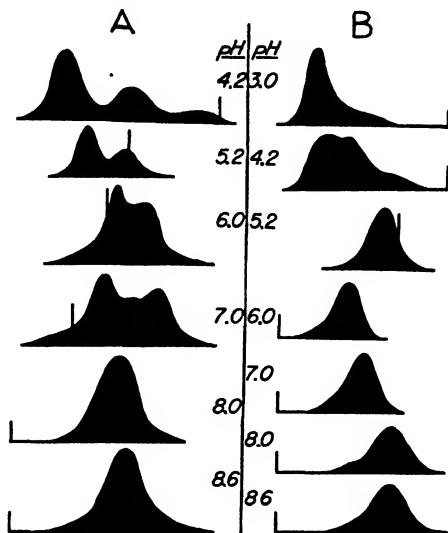


FIG. 5. Effect of pH on electrophoretic behavior of milk lactoglobulins. A. Goat lactoglobulin. Duration of all experiments was 7200 seconds. B. Bovine lactoglobulin. Duration of experiments at pH 3.0, 7.0, 8.0, and 8.6 was 5400 seconds. All other experiments 7200 seconds.

one component is present at pH values of 8.0 and above, while at a pH of 7.0, three components appear. At pH values of less than 7.0 there are at least 2 components observable, but, in some experiments, the third has been more or less obscured. In Fig. 6, mobilities have been plotted as a function of pH, and, for the goat lactoglobulin, it may be seen that component No. 1 has an isoelectric point of 5.9, while component No. 2 has an isoelectric point of 5.2. The complete curve for component No. 3 could not be followed.

In the case of bovine lactoglobulin the effect of varying pH on the electrophoretic patterns is shown in Fig. 5B. This particular product, when studied, contained about 7% of a slower migrating impurity at

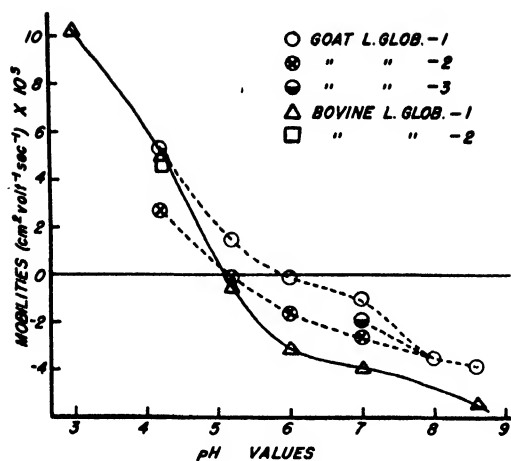


FIG. 6. pH-mobility curves for milk lactoglobulins.

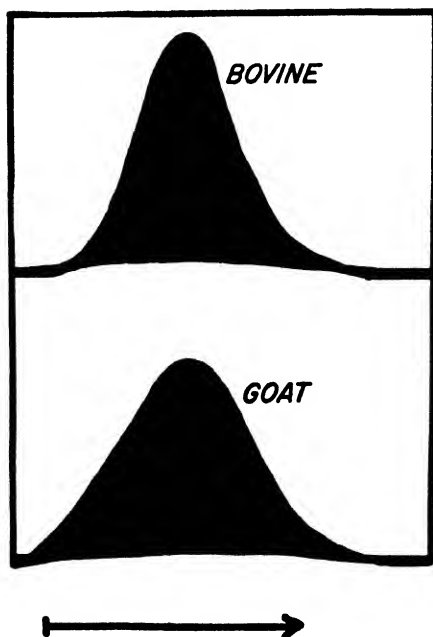


FIG. 7. Sedimentation patterns for milk lactoglobulins taken after 100 minutes at 50,400 r.p.m.

pH 8.6 which should be taken into account in interpreting the patterns. There is no apparent electrophoretic splitting at pH values of 5.2 and above. However, at pH 4.2 a definite split into two components has occurred. At pH 3.0 the peak is still asymmetrical, but the two components have again almost merged into one. That no irreversible change in the protein at low pH values had occurred was shown by the fact that a sample could be subjected to the usual dialyses at pH 4.2, and then redialyzed at pH 8.6, to give a single symmetrical peak upon electrophoresis at the higher pH. This reversibility was also demonstrated with the goat lactoglobulin. From Fig. 6 it can be seen that the bovine lactoglobulin is isoelectric at pH 5.1, in close agreement with the data of Pedersen (12) and of Cannan, Palmer and Kibrick (13) who found 5.18 for the pH of zero net charge.

Both the bovine and goat lactoglobulin preparations sedimented as single components. The goat lactoglobulin gave an average value of  $S_{20W} = 3.0$  Svedberg units but showed far more spreading during sedimentation than could be attributed to diffusion. The bovine lactoglobulin ( $\beta$ -lactoglobulin) gave a value of  $S_{20W} = 3.2$  Svedberg units which compares well with Pedersen's finding of  $S_{20W} = 3.12$  Svedberg units (12). No increase in the apparent diffusion constant during the course of sedimentation occurred which shows that, molecularly, the bovine  $\beta$ -lactoglobulin preparations were essentially monodisperse.

## DISCUSSION

These studies illustrate the application of the low temperature ethanol fractionations to milk whey protein systems. The advantages of this type of fractionation have been pointed out by Cohn *et al.* (2).

The other point which is emphasized is the necessity for studying a given electrophoretic entity over a wide pH range before drawing any conclusions as to its electrophoretic purity. Although this point has been emphasized previously (14), numerous examples exist where such measurements were not made.

The failure to crystallize either of the lactoglobulins studied from concentrated salt solutions may be due to the ethanol treatment, although this seems unlikely, since Berger *et al.* (15) have been able to crystallize yeast hexokinase by such methods following ethanol fractionation.

## ACKNOWLEDGMENT

The authors wish to thank Mr. E. M. Hanson for carrying out the sedimentation experiments, and to acknowledge the interest of Dr. J. W. Williams during the course of this investigation.

## SUMMARY

The ethanol fractionation has been applied to the milk whey proteins of the cow and goat. Lactoglobulins showing one peak upon electrophoresis at a given pH were obtained in good yield. These proteins, however, showed the presence of more than one component when pH mobility curves were determined. Both preparations showed one component on sedimentation analysis but only the bovine lactoglobulin appeared to be molecularly homogeneous.

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## Pantothenic Acid Studies. II. The Influence of Glutamic Acid and Pantoic Acid on Yeast Growth<sup>1,2</sup>

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Received August 4, 1947

### INTRODUCTION

$\beta$ -Alanine is generally regarded as a precursor of pantothenic acid for the growth of yeasts, and is believed to be converted to the complete vitamin by coupling with pantoic acid.

Utilization of the  $\beta$ -alanine moiety for growth, however, is frequently far less efficient than that of pantothenic acid. It has been reported that  $\beta$ -alanine is toxic to yeast except when asparagine or aspartic acid is a constituent of the medium (1).  $\beta$ -Aminobutyric acid, phenyl- $\beta$ -alanine and isoserine neutralize the growth-promoting action of  $\beta$ -alanine on yeast, although they have no effect when pantothenic acid is used (2). Previous work in this laboratory (3) has shown that several amino acids may retard growth stimulation by  $\beta$ -alanine, although not by pantothenic acid. On the other hand, ammonium ion at proper concentration can enhance the utilization of  $\beta$ -alanine but not pantothenic acid (4).

Since the utilization of  $\beta$ -alanine by yeast is influenced by other substances in the medium, it was felt that these might be employed to study the biosynthetic processes involving pantothenic acid. The present work is a comparison of the effect of glutamic acid and pantoic acid on the utilization of pantothenic acid and  $\beta$ -alanine in a synthetic medium by various yeasts.

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<sup>1</sup> For the first paper in this series, see Cheldelin, V. H., and Schink, C. A., *J. Am. Chem. Soc.* **69**, 2625 (1947).

This work was supported by the Nutrition Foundation, Inc., and the National Institute of Health.

<sup>2</sup> Published with the approval of the Monograph Publications Committee, Oregon State College. Research Paper No. 112, School of Science, Department of Chemistry.

## EXPERIMENTAL

The testing method was the same as reported in a previous paper (3). The medium used previously was adapted to the needs of the present study, so that the effect of various dicarboxylic acids could be observed. The composition is shown Table I.

TABLE I

*Basal Medium for Yeast Growth*

Glucose	20 g.
Ammonium sulfate	3 g.
Potassium dihydrogen phosphate	2 g.
Salt solution 1 and 2 (3)	1 ml. each
Adenine sulfate	20 mg.
Inositol	15 mg.
Riboflavin	200 $\gamma$
Nicotinic acid	200 $\gamma$
Pyridoxine	200 $\gamma$
<i>p</i> -Aminobenzoic acid	200 $\gamma$
Biotin	1 $\gamma$
Folic acid	2 $\gamma$
Thiamine chloride	100 $\gamma$
Water to	1 liter
pH	4.8-5.0

## RESULTS

The effect of various levels of glutamic acid was tested for four yeasts<sup>3</sup> in the presence of 0.1  $\gamma$  pantothenic acid or 0.2  $\gamma$   $\beta$ -alanine per tube. All showed enhanced growth when the level of glutamic acid was less than 1 mg. per tube in the presence of 0.1  $\gamma$  of pantothenic acid. Two strains, LM<sup>3</sup> and 2504,<sup>3</sup> were stimulated even when glutamic acid was present at a level of 2 mg. per tube, while the other two strains were inhibited at the higher level of glutamic acid. In the presence of 0.2  $\gamma$   $\beta$ -alanine, glutamic acid showed increased growth in strains LM and 2504 at all levels of glutamic acid. One-half mg. per tube appeared to be optimum. Growth in these tubes was nearly as effective as an equivalent amount of pantothenic acid. Table II illustrates the effects

<sup>3</sup> The four yeast strains used herein are numbered according to a system used previously (3). In addition, the American Type Culture Collection has assigned them the following numbers: *S. cerevisiae*, S.C. strain, No. 9369; strain 2504, No. 9370; LM strain, No. 9371; and *S. carlsbergensis* 1036, No. 9373. Strain 1036, obtained from Dr. C. N. Frey, Fleischmann Laboratories, has been listed previously under the number 4228 (3). Cultures of these four strains may be obtained from the American Type Culture Collection.

TABLE II

*Growth Response of Yeasts to Different Levels of Glutamic Acid at Suboptimum Levels of  $\beta$ -Alanine or Pantothenic Acid <sup>a</sup>*

Turbidimetric readings<sup>b</sup>

Yeast strain <sup>3</sup> Constituents per tube	S. C.	1036	LM	2504
$\beta$ -Alanine (Standard)				
0.0 $\gamma$	0.015	0.015	0.008	0.030
0.2	0.335	0.235	0.055	0.190
2.0	0.480	0.285	0.260	0.240
0.2 $\gamma$ $\beta$ -Alanine + glutamic acid				
0.5 mg.	0.050	0.030	0.280	0.280
1.0	0.030	0.050	0.250	0.270
2.0	0.030	0.025	0.238	0.260
5.0	0.030	0.010	0.100	0.210
Pantothenic acid (Standard)				
0.1 $\gamma$	0.175	0.115	0.100	0.235
0.2	0.350	0.225	0.225	0.240
2.0	0.470	0.285	0.265	0.220
0.1 $\gamma$ Pantothenic acid + glutamic acid				
0.5 mg.	0.245	0.120	0.190	0.285
1.0	0.195	0.140	0.165	0.300
2.0	0.150	0.080	0.138	0.280
5.0	0.090	0.030	0.075	0.245

<sup>a</sup> All amounts of pantothenic acid in this paper are expressed in terms of calcium pantothenate.

<sup>b</sup> All turbidimetric values are given in terms of optical density (equals 2 minus log G).

of different glutamic acid levels at suboptimum levels of  $\beta$ -alanine and pantothenic acid, whereas Table III summarizes the effect of 0.5 mg. glutamic acid per tube at different levels of  $\beta$ -alanine or pantothenic acid.

When glutamic acid was fixed at 0.5 mg. per tube its stimulatory effect on yeast was exhibited at any level of pantothenic acid, but was most distinctive at the lower concentrations. On the other hand,

TABLE III

*Growth Response of Yeasts to Different Levels of  $\beta$ -Alanine or Pantothenic Acid in the Presence of 0.5 mg. Glutamic Acid per Tube*

Turbidimetric readings			
Yeast strain <sup>a</sup> Constituents per tube	S. C.	1036	LM
Pantothenic acid (Standard)			
0.0 $\gamma$	0.025	0.030	0.020
0.1	0.140	0.150	0.140
2.0	0.580	0.400	0.430
0.5 mg. Glutamic acid + pantothenic acid			
0.1 $\gamma$	0.025	0.180	0.310
2.0	0.630	0.450	0.550
$\beta$ -Alanine (Standard)			
0.2 $\gamma$	0.310	0.390	0.090
2.0	0.550	0.320	0.310
0.5 mg. Glutamic acid + $\beta$ -alanine			
0.2 $\gamma$	0.030	0.030	0.300
2.0	0.580	0.360	0.560

except with strain LM, 0.5 mg. of glutamic acid suppressed the growth-promoting property of lower levels of  $\beta$ -alanine, as shown in Table II.

To determine whether the inhibitory action of glutamic acid on *S. carlsbergensis* 1036<sup>a</sup> (as well as several strains of *S. cerevisiae* tested) might be due to competition between pantoic acid and glutamic acid for conjugation with  $\beta$ -alanine, growth was noted with varying amounts of pantoic acid. This is summarized in Table IV for two yeasts.

In the absence of glutamic acid, pantoic acid in moderate concentration stimulated strain 1036 slightly, but had no effect on the growth of LM. This can be explained by assuming that the rate of synthesis of pantoic acid by 1036 is not as rapid as the coupling of pantoic acid with  $\beta$ -alanine. However, an excess of pantoic acid inhibited growth at all levels of  $\beta$ -alanine, and the inhibitory action of glutamic acid on 1036

could not be released by the addition of a 10-fold excess of pantoic acid. This inhibition, therefore, cannot be due to conjugation of glutamic acid with pantoic acid. Further, the stimulatory action of glutamic acid on LM yeast was not enhanced by the addition of pantoic acid to the medium; instead, a high concentration of pantoic acid caused an 80% reduction in growth.

Glutamic acid and pantoic acid were both found (Table IV) to be without activity when tested in the absence of  $\beta$ -alanine or pantothenic acid. The stimulatory effect of these substances, therefore, cannot be due to their activity *per se*.

TABLE IV  
*Effect of Pantoic Acid and Glutamic Acid On Yeast Growth*

Yeast strain <sup>a</sup> Constituents per tube	Turbidimetric readings	
	1036	LM
<i><math>\beta</math>-Alanine (Standard)</i>		
0.0 $\gamma$	0.015	0.000
0.2	0.185	0.040
1.0	0.320	0.345
5.0	0.360	0.460
0.2 $\gamma$ <i><math>\beta</math>-Alanine +</i>		
<i>pantoic acid</i>		
0.01 mg.	0.170	0.045
0.10	0.225	0.045
1.00	0.150	0.055
5.00	0.100	0.050
0.2 $\gamma$ <i><math>\beta</math>-Alanine</i>		
<i>+0.5 mg. glutamic acid</i>		
	0.000	0.290
0.2 $\gamma$ <i><math>\beta</math>-Alanine + 0.5 mg.</i>		
<i>glutamic acid + pantoic</i>		
<i>acid</i>		
0.01 mg.	0.005	0.300
0.10	0.012	0.310
1.00	0.007	0.275
5.00	0.005	0.075
0.5 mg. <i>Glutamic acid +</i>		
<i>pantoic acid</i>		
0.10 mg.	0.000	0.005
5.00	0.000	0.005
Pantoic acid		
0.00 mg.	0.000	0.000
0.10	0.000	0.000
5.00	0.000	0.000

The experiments in Table II were repeated in a medium which had been sterilized by filtration. The results were the same as noted above, so it may be concluded that the effect of glutamic acid is not due to the formation of other growth substances during heating.

## DISCUSSION

The literature dealing with yeast nutrition during the past 30 years has often contained contradictory material. This is probably due mainly to differences in the strains of yeast employed. Two such observations have been made by workers at the Carlsberg Laboratories which do not agree with those presented here. Nielsen and Johansen (5) from an experiment on "Hefestamm C.L. 1" concluded that when either or both glutamic acid and thiamine are added to the medium, growth-promoting action is increased and that of  $\beta$ -alanine becomes as great as that of pantothenic acid. Similar experiments in this laboratory, however, indicate that thiamine is not directly concerned with the  $\beta$ -alanine-pantothenic acid system. Growth in the absence of thiamine is attained equally by  $\beta$ -alanine and pantothenic acid, and an additional growth effect is produced by thiamine, which is of equal magnitude for both  $\beta$ -alanine and pantothenic acid.

Hartelius and Johansen (6) observed further that, in the presence of suboptimum amounts of  $\beta$ -alanine, excess pantoic acid approximately doubled yeast growth, and provided a possible stimulation even with optimum levels of  $\beta$ -alanine and all levels of pantothenic acid. Our findings, as already noted, do not agree with these.

The stimulatory effect of glutamic acid (as well as aspartic acid and asparagine) in the presence of suboptimal amounts of pantothenic acid or  $\beta$ -alanine does not seem to occur commonly among yeasts. Previously (3) asparagine had been found to curtail growth in 14 of 16 strains of *S. cerevisiae* and one strain of *S. carlsbergensis*, whereas only strains 2504 and LM resisted curtailment. The reasons for the inhibitory effects are difficult to evaluate, since several other amino acids also exhibit this property,<sup>4</sup> but the utilization of  $\beta$ -alanine is undoubtedly involved, inasmuch as these effects are not observed when pantothenic acid is the growth factor.

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<sup>4</sup> King, T. E., and Cheldelin, V. H., unpublished observations.

The stimulatory action of 0.5 mg. of glutamic acid on the growth of yeasts LM and 2504 cannot be due to the extra nitrogen added, nor can it be explained by simple decarboxylation, since aspartic acid is less effective.<sup>4</sup> If glutamic acid were oxidized by  $\beta$ - or  $\omega$ -oxidation to form  $\beta$ -alanine, which, in turn, could couple with pantoic acid to form more pantothenic acid, then glutamic acid should show some stimulatory action in the absence of pantothenic acid or  $\beta$ -alanine. Auto-catalysis also seems unlikely, since very small amounts (0.01  $\gamma$ ) of pantothenic acid or  $\beta$ -alanine were ineffective in the presence of glutamic acid (data not shown). Moreover, after 96 hours incubation glutamic acid improved the growth in the presence of either nutilite.

The results expressed in Table IV have shown that glutamic acid probably does not combine with pantoic acid, nor is it a growth factor for yeast itself. The most favorable explanation of the role of glutamic acid seems at present to be that, under the influence of yeast cells, it combines with pantothenic acid or  $\beta$ -alanine (and possibly with pantoic acid in the proper linkage) to produce a substance which is more active for the growth of yeast. Further studies are in progress to determine the character of such combined forms.

#### ACKNOWLEDGMENT

The technical assistance of Ruth S. Langdon is gratefully acknowledged.

#### SUMMARY

The effect of glutamic acid on the growth of four strains of yeasts has been studied.

1. Growth of all four strains of yeast tested was enhanced by 1 mg. or less of glutamic acid in suboptimum concentrations of pantothenic acid.

2. In the presence of suboptimum concentrations of  $\beta$ -alanine, *S. cerevisiae* "S. C." strain and *S. carlsbergensis* were inhibited by glutamic acid, whereas strains LM and 2504 were enhanced.

3. Pantoic acid exhibited a slight growth effect upon strain 1036 at suboptimum levels of  $\beta$ -alanine, but not in the presence of adequate amounts of  $\beta$ -alanine or at any level of pantothenic acid. Excess pantoic acid inhibited growth of all yeasts.

4. The mechanism of glutamic acid action has been discussed briefly.



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# Adenylpyrophosphatase Activity in Epidermal Carcinogenesis in Mice\*

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Received August 4, 1947

## INTRODUCTION

The object of this study was to determine the changes in adenylpyrophosphatase activity in mouse epidermis undergoing epidermal carcinogenesis and to correlate the results with the previously reported (12) changes in cytochrome oxidase and succinic dehydrogenase activity as part of our program on the integration of the chemical, physical, and histological changes in epidermal carcinogenesis produced by a rigidly standardized procedure (13).

Adenosinetriphosphate plays a central role in the energy metabolism of the cell (1,2). Evidence for the importance of enzymes hydrolyzing ATP in the cellular economy has recently been summarized (3, 4, 5). There is support for the view that the balance between ATP and products of its hydrolysis may be important in the regulation not only of glycolysis and respiration, but also of fat and protein metabolism. Apyrase (an abbreviation for adenylpyrophosphatase), cytochrome oxidase, and succinic dehydrogenase activities have been localized to a large extent in particulate matter within cells, the so-called large granule fraction, which is believed to be closely associated with the secretory granules and mitochondria (6, 7, 8, 9). The total apyrase activity of homogenates of liver and hepatic tumors was found to be approximately the same (10, 11), but there was a significant alteration from the normal in the distribution of the enzymatic activity between different cellular fractions in the tumor tissue (11). This indicates the

\* Aided by grants from the U. S. Public Health Service and the Charles F. Kettering Foundation.

possibility of an alteration in the activity *in vivo* of the enzymes hydrolyzing ATP.

## EXPERIMENTAL

### *Materials and Methods*

Swiss mice of both sexes were painted with methylcholanthrene (0.6% by wt./vol. in reagent grade benzene or with benzene alone for controls). The experimental mice were sacrificed 5 days after the last application of either benzene or carcinogen, and the control mice 5 days after the backs were shaved. Epidermis almost free of dermis was removed rapidly by scraping at room temperature (14). Papillomata were easily detached from the surrounding tissue. The tumor studied was a transplantable squamous cell carcinoma (15) originally derived from a carcinoma produced by the application of methylcholanthrene to the skin of a mouse and now in approximately the fiftieth transplantation. The analyses were limited to very small young tumors from which the connective tissue capsule and the small amount of central necrotic material was removed.

All tissues studied were chilled in weighing bottles in an ice bath immediately after removal, weighed rapidly on a torsion balance, homogenized in a ground-glass homogenizer in ice-cold redistilled water, and diluted to a tissue concentration of 1%. Apyrase activity was determined by the assay method of Dubois and Potter (16). It was possible to employ somewhat smaller quantities of ATP than suggested by the latter authors because of the generally lower activity of the tissues studied. The incubation mixture in the standard assay procedure usually contained 47–53  $\gamma$  of labile P as the sodium salt of ATP or 39  $\gamma$  of labile P as the sodium salt of ADP. The labile P was that liberated by hydrolysis for 10 minutes at 100°C. in 1 *N* sulfuric acid, and was determined for each sample of substrate employed. Highly purified barium salts of ATP and ADP were kindly supplied to us by Drs. G. A. LePage and H. A. Lardy of the Univ. of Wisconsin. These were converted to the sodium salts before use by the addition of a 10% excess of sodium sulfate. In all determinations, incubation was carried out under identical conditions, with and without substrate, and the P found in the sample without substrate was subtracted from that found in the tube containing substrate. A correction was also made for the inorganic phosphate in the substrate solution. It was established that, under none of the experimental conditions employed in this study, were appreciable quantities of phosphate liberated from ATP in the absence of enzyme. The incubation was carried out at 38°C. for 15 minutes in 5 ml. volumetric flasks attached to rubber tubing nailed to a wooden frame mounted on a Warburg shaker. This allowed 3-dimensional shaking and avoided any settling of the homogenate during the incubation. The incubation mixture (total volume, 0.68 ml.) was deproteinized by the addition of 0.07 ml. of 50% ice-cold trichloroacetic acid. The flasks were centrifuged in a refrigerated centrifuge at 0°C. and the supernatant was poured off carefully for analysis. All inorganic phosphate analyses for the experiments carried out by the standard assay procedure were performed by the Lowry and Lopez (17) method in the Coleman Spectrophotometer, Model 10S, at a wavelength of 700 m $\mu$ . The Truog and Meyer (18) procedure was employed for the determination of labile phosphate and for the determination of inorganic phosphate

in the studies of the time relationships of the action of the tissues on ATP and ADP. Suitable multiples of the quantities of solutions employed in the standard assays were used in the experiments performed on a larger scale. In all of the latter experiments the final tissue concentration expressed on a fresh weight basis was 15 mg.-%.

The liberation of phosphate from ATP by normal epidermis was linear for 20 minutes, but the rate decreased somewhat at 30 minutes under conditions in which substrate was not a limiting factor. Therefore, the 15 minute period of incubation originally suggested (16) was employed.

For samples of normal and hyperplastic epidermis and papillomata the quantity of phosphate liberated from ATP was proportional to the amount of tissue when 0.1, 0.2 or 0.3 ml. of the same homogenate was employed. However, in the case of carcinomata the response was not always proportional to the tissue concentration, but was sometimes relatively greater for 2 and 3 mg. than 1 mg. of tissue. There might be better protection against the destruction of the enzyme systems involved by greater tissue concentrations or some activator might be limiting at lower tissue concentrations. In this connection it should be noted that a given fresh weight of tumor contains only approximately one-half of the dry weight that a similar quantity of normal or hyperplastic epidermis contains (14). The activities reported for the tumor tissue in this study are thus probably minimal values. The activity at different tissue concentrations was not determined for all samples assayed. In many of the determinations homogenate equivalent to 3 mg. of tissue was employed and the results expressed in  $\gamma$  of P liberated from the substrate in 15 minutes/mg. of fresh weight of tissue. Activation by the calcium ion was observed in all instances studied. Results obtained with hyperplastic epidermis and papillomata were virtually identical with those obtained for normal epidermis. Values similar to those with ATP were obtained for all tissues when ADP was used as substrate.

## RESULTS

### *Liberation of Inorganic P from ATP and Sodium- $\beta$ -Glycerophosphate by Normal Epidermis and Carcinoma (Table I)*

Dubois and Potter (16) demonstrated under their experimental conditions that the liberation of phosphate from ATP did not result from the action of a nonspecific phosphatase. A similar experiment was performed in the present studies, in which the action of normal epidermis and carcinoma on ATP and  $\beta$ -glycerophosphate were compared. From the results in Table I it is evident that there was little or no activity of the homogenate of either tissue on the glycerophosphate and there was definite liberation of phosphate from ATP. It is also apparent that carcinoma has much more apyrase activity than normal epidermis. The activity of normal epidermis is lower than that of any of the tissues previously reported (10, 16), an observation consonant with the extremely low values found for succinic dehydrogenase and

cytochrome oxidase (12). The activity of the carcinoma, however, is in the range previously reported for a variety of neoplasms (10).

TABLE I

*Liberation of Inorganic Phosphorus from ATP and Na<sub>2</sub> β-glycerophosphate, 5½ H<sub>2</sub>O, by Aliquots of the Same Tissue Samples (1.3 × 10<sup>-6</sup> moles of substrate per tube)*

Sample No.	ATP	Glycerophosphate
	Normal epidermis	
	γ P/mg.	γ P/mg.
16-N	2.6	0.0
17-N	2.1	0.0
18-N	1.8	0.0
19-N	1.7	0.0
Mean	2.1	0.0
	Carcinoma	
13-C	10.0	0.1
14-C	7.4	0.0
15-C	9.3	0.1
Mean	8.9	0.1

*Time-Course of Liberation of Phosphate from ATP and ADP*

Preliminary results indicated that tumor homogenates could probably split all three phosphate groups from ATP in experiments employing the standard assay procedure in which there were employed quantities of ATP that contained phosphate approaching the limits of accuracy of the analytical procedure. However, similar experiments with normal epidermis gave highly variable results. Experiments were then performed on a larger scale in which the concentrations of ATP and ADP were small, but from which sufficient quantities could be removed for accurate analysis. The time-course of liberation of inorganic phosphate by homogenates of normal and hyperplastic epidermis and tumor was determined. The effect of calcium ions on this process was studied in each type of tissue and the influence of added citrate, anaerobiosis, and magnesium ions was studied in the case of tumors.

The results of all of the experiments are consistent with the interpretation that enzymes which can completely dephosphorylate ATP are present in the homogenates of all of the tissues studied. This has been shown to be also the case for brain (19, 20), embryonic tissue, liver, and Jensen's sarcoma of rats (21). A specific enzyme capable of dephosphorylating myoadenylic acid has been discovered in animal tissues by Reis (22).

Fig. 1 shows the curves of liberation of phosphate by calcium-activated homogenates of tumor and normal epidermis in experiments performed simultaneously. Both tissues liberated all of the phosphate in ATP. However, the tumor homogenate brought the reaction to completion in 15 minutes, while an equivalent amount of normal tissue (on a fresh weight basis) accomplished this in 45 minutes, a difference

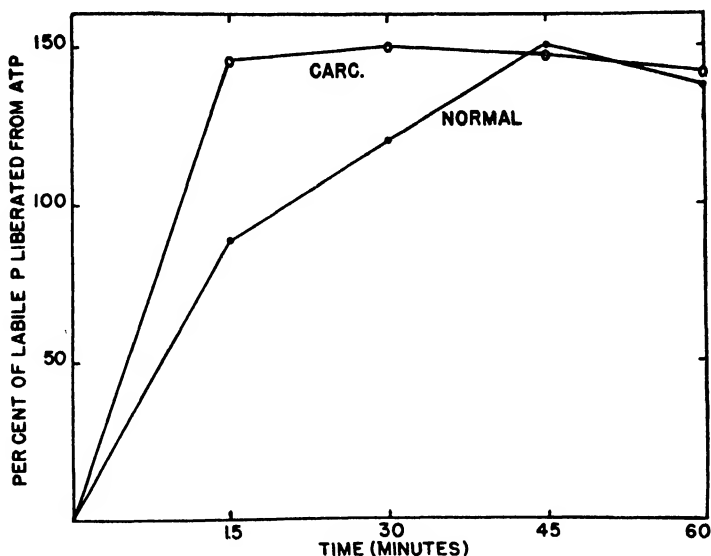


FIG. 1. Liberation of P from ATP by normal epidermis and squamous cell carcinoma. Total volume, 34 ml.; 47.3  $\gamma$  of labile P as ATP.

made even more impressive when it is recalled that 81.6% of the fresh weight of these tumors is water, while only 60% of normal epidermis is water (14). The results of a similar experiment in which ADP was employed as substrate are shown in Fig. 2. Also, in this instance, all of the phosphate was liberated as inorganic phosphate in 15 minutes by tumor homogenates, while the reaction took 45 minutes with normal epidermis. On the basis of the above experiments it appears that tumor tissue is approximately 3 times as active as normal epidermis in liberating P from ATP on a wet weight basis and 6 times as active on a dry weight basis. These results were confirmed by experiments to be discussed in another section of this paper in which substrate concentration was not a limiting factor. It is interesting to note that the quantity of

inorganic phosphate fell in the case of tumor hydrolysis of ADP (Fig. 2) after the maximal possible value had been attained. Several other experiments in which ATP was the substrate have given similar results. It is not possible at the present time to make a statement concerning the nature of this slow removal of phosphate.

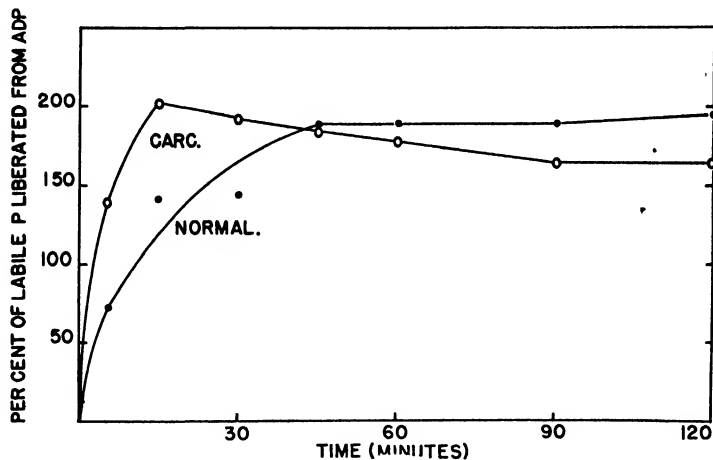


FIG. 2. Liberation of P from ADP by normal epidermis and squamous cell carcinoma. Total volume, 51 ml.; 38.1  $\gamma$  of labile P as ADP.

Another experiment in which the hydrolysis of ATP by normal epidermis was studied is shown in Fig. 3. The hydrolysis was more rapid in the presence of calcium. Similar results are shown for tumor in Fig. 5. In these experiments the reaction was brought to completion by the tumor homogenate 3 times as rapidly as by the normal epidermis when calcium was present and twice as rapidly in the absence of calcium. Activation by calcium is also apparent in the hyperplastic epidermis obtained from mice painted 18 times with methylcholanthrene (Fig. 4). However, the hydrolysis by the hyperplastic epidermis was slower than that by normal epidermis, the homogenate with calcium failing to bring the reaction to completion in 60 minutes. In the absence of calcium a quantity of phosphate was liberated which accounted for the hydrolysis of less than 2 or the 3 phosphate groups. Results obtained under the standard assay conditions showed no significant difference between hyperplastic and normal epidermis. The difference in the above experiment may possibly lie in the activity of

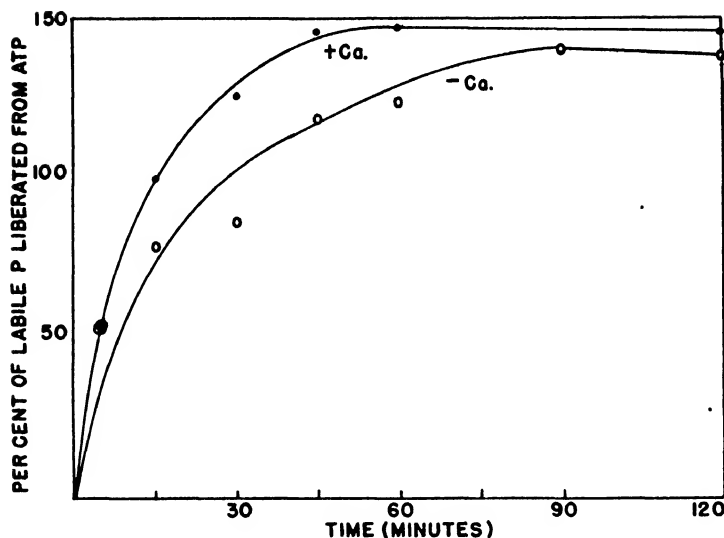


FIG. 3. Liberation of P from ATP by normal epidermis in the presence or absence of added Ca. Total volume, 51 ml.; 53.5  $\gamma$  of labile P as ATP.

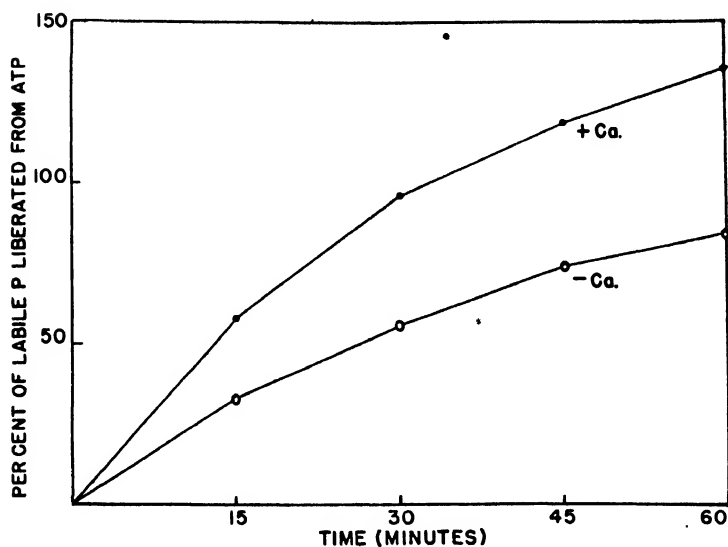


FIG. 4. Liberation of P from ATP by hyperplastic epidermis in the presence or absence of added Ca. Total volume, 34 ml.; 47.3  $\gamma$  labile P as ATP.



the enzyme system catalyzing the hydrolysis of the adenylic acid resulting from the dephosphorylation of ATP. This system may not contribute importantly to the total quantity of phosphate liberated from ATP or ADP in the standard assay procedure when the concentration of the substrate is relatively high and the period of incubation short. This is supported by the fact that the decrease in 10 minute-hydrolyzable P could account for almost all of the P liberated in the assay procedure by normal and hyperplastic epidermis and tumors

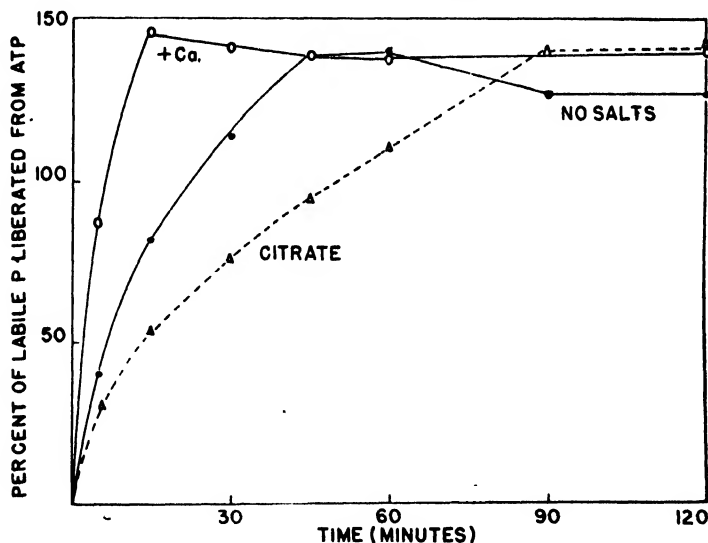


FIG. 5. Liberation of P from ATP by squamous cell carcinoma with or without added Ca or with added citrate ( $2.0 \times 10^{-3} M$ ). Total volume, 51 ml.; 69.6  $\gamma$  of labile P as ATP.

(see Table II). It would be interesting to determine whether the adenylic acid formed during the dephosphorylation of ATP and ADP is hydrolyzed further by the same enzyme system as the parent compounds, or is first deaminated to inosinic acid and then dephosphorylated, or is attacked by a nonspecific phosphatase or by a specific 5-nucleotidase.

Results of studies with tumor homogenates under different conditions are shown in Figs. 5 and 6. The effects of calcium and citrate on the hydrolysis of ATP are shown in Fig. 5. As in the previous experiments, activation by calcium is evident. Citrate, in a concentration of  $2 \times 10^{-3} M$ , decreased the rate of hydrolysis considerably below

that of the unactivated homogenate. The citrate ion probably acts by forming complexes with calcium and magnesium ions present in the homogenate. In the anaerobic experiment (Fig. 6) the magnesium was present in a concentration equivalent to that of the calcium, since it was found that magnesium is as effective as calcium in activating the system responsible for the breakdown of ATP. The two ions together had no greater effect than that expected from either one alone. Anaerobiosis and lowering the temperature to 18.5°C. produced no qualitative change in the hydrolysis by the activated preparation or by the samples containing no activator. However, in this

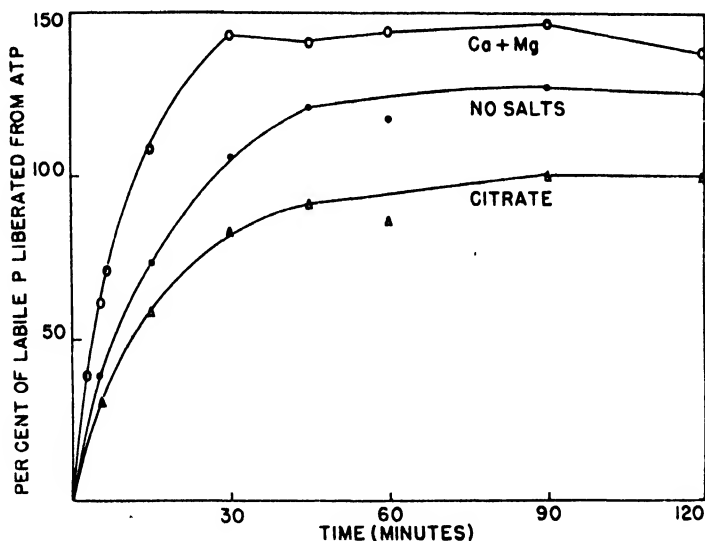


Fig. 6. Liberation of P from ATP by squamous cell carcinoma in presence of Ca and Mg, with no salts added, or with added citrate ( $2.8 \times 10^{-3} M$ ). Anaerobic ( $N_2$ ); 18.5°C.; total volume, 100 ml.; 200  $\gamma$  of labile P as ATP.

experiment the mixture containing the citrate ( $2.8 \times 10^{-3} M$ ) had liberated phosphate in quantities equivalent to only 2 of the 3 phosphate groups. These findings suggest that the homogenate incubated aerobically may have been able to remove citrate either by oxidation or by some other metabolic conversion. The difference in the degree of phosphorolysis observed in the anaerobic experiment suggests that citrate may inhibit the system operating in the degradation of the myoadenylic acid. Citrate was also consistently inhibitory when employed in the standard assay procedure. The effect of citrate is interesting in connection with the high citrate content observed in tumors by Dickens (23, 24). Citrate may be an important substance in regulating *in vivo* the apyrase activity as well as that of other enzymes the maximal activity of which depends on ions with which citrate can form complexes.

*Changes in 10 Minute-Hydrolyzable P and Inorganic P in the Standard Assay Procedure*

It was necessary to determine whether the measurement of inorganic P liberated from ATP was a valid measure of the breakdown of ATP, since it was conceivable that removal of the phosphate liberated from ATP could be taking place by esterification, transphosphorylation, or by some other process. Results of experiments on normal and hyperplastic epidermis and carcinomata are shown in Table II. In the case

TABLE II  
*Comparison of Changes in 10 Minute-Hydrolyzable P and Inorganic P in the Standard Assay Procedure*

Sample	$\gamma$ of P/mg. of fresh tissue	
	$\Delta P^{10}$ $\gamma$	$\Delta$ Inorganic P $\gamma$
Normal epidermis		
1-N	-4.3	+2.7
2-N	-3.7	+3.3
3-N	-4.5	+2.5
4-N	-3.8	+2.3
5-N	-4.7	+2.9
6-N	-3.9	+2.4
Mean	-4.2	+2.7
Hyperplastic epidermis (12 paintings)		
1-H	-3.9	+1.3
2-H	-4.1	+2.7
3-H	-3.7	+2.8
4-H	-3.8	+3.1
5-H	-2.3	+1.9
6-H	-3.5	+3.7
Mean	-3.6	+2.6
Squamous cell carcinoma		
1-C	-9.6	+9.4
2-C	-7.0	+6.9
3-C	-6.2	+6.7
4-C	-5.7	+6.7
5-C	-7.9	+8.4
Mean	-7.3	+7.6

of tumors, the results for decrease in 10 minute-hydrolyzable P were virtually identical with those for the increase in inorganic P. There was a slightly greater decrease in 10 minute-P in the normal and hyperplastic epidermis than could be accounted for by the increase in inorganic P. These differences were so small that the increase of inorganic

P was considered to be a valid index of the liberation of P. It is likely that the 10 minute-P values were in slight error because of some adsorption of ATP on the precipitate formed during deproteinization. The quantity of precipitate was larger in the case of normal and hyperplastic epidermis than in tumors. There were negligible quantities of 10

TABLE III  
*Liberation of Inorganic P from ATP and ADP by Aliquots  
of the Same Tissue Samples*

Sample number	Apyrase activity	
	ATP	ADP
	Normal epidermis	
	$\gamma$ P/mg.	$\gamma$ P/mg.
7-N	4.1	4.3
8-N	4.1	3.6
9-N	3.8	3.5
10-N	5.0	4.4
11-N	3.9	3.2
12-N	2.2	2.6
13-N	1.9	1.8
14-N	1.3	2.3
15-N	2.4	3.1
Mean	3.2	3.1
	Hyperplastic epidermis	
3 paintings with MC*	1.9	2.0
6 paintings with MC	3.1	3.1
12 paintings with MC	5.4	4.7
24 paintings with MC	3.3	2.6
Mean	3.4	3.1
	Papilloma	
1-P	3.2	3.7
2-P	2.9	2.7
3-P	4.5	3.8
4-P	1.9	2.7
5-P	5.6	4.5
Mean	3.6	3.5
	Carcinoma	
6-C	14.7	10.5
7-C	9.1	7.8
8-C	11.7	10.3
9-C	12.1	11.8
10-C	10.0	9.7
11-C	9.8	9.0
12-C	5.9	5.6
Mean	10.5	9.2

\* Methylcholanthrene.

minute-P in the dilute homogenates employed. The above results again show the greater activity of carcinoma and emphasize the similarity in the activities of normal and hyperplastic epidermis. It appears unlikely that under the conditions of the standard assay the P liberated from adenylic acid played an important role, since in no case was the mean of the inorganic values significantly greater than that of the 10 minute-P.

#### *Comparison of the Activity of Tissues on ATP and ADP*

It was of interest to compare the liberation of inorganic P from ATP and ADP in view of the previously discussed experiments in which it was shown that homogenates of both normal epidermis and tumors could remove all the P from both substrates and that the action of tumors was more rapid than that of an equivalent amount of normal tissue. Kalckar (25) showed that the same enzyme probably hydrolyzes both ATP and ADP in liver preparations, and Potter (3) quoted unpublished experiments as indicating that ADP as well as ATP could be hydrolyzed in his experiments. The results in Table III show a consonance in the quantities of inorganic P liberated from both substances by aliquots of the same homogenates in almost all of the tissues studied. In tumors the activity was slightly greater with ATP as substrate. However, even in them the consonance was notable. Although this result is not proof of the identity of the enzyme systems hydrolyzing ATP and ADP, it is certainly indicative of their close association. The mean activity of tumor homogenates was approximately 3 times greater on both substrates than that of the other tissues studied. Normal and hyperplastic epidermis and papillomata gave closely similar mean values for activity with either substrate.

#### *Summary of Assay Results*

The summary of all the assays performed on the samples of epidermis and tumors in epidermal carcinogenesis is contained in Table IV. No significant changes in apyrase activity occurred in the precancerous stages, but a highly significant increase took place in the carcinoma. The mean value for tumors was 3 times that observed for normal epidermis on the basis of wet weight and approximately 6.5 times on the basis of dry weight. The lowest value for the 19 samples of carcinomata studied was higher than the highest value obtained for any of the

other 47 samples examined. The values for dry weights were obtained from a previous study (14). A number of determinations of water content performed in the present experiments were in agreement with those previously reported.

TABLE IV  
*Liberation of Inorganic P from ATP in Epidermal Carcinogenesis*  
Summary

Tissue	Number of determinations	Apyrase activity			
		$\gamma$ P/mg. fresh weight		$\gamma$ P/mg. dry weight <sup>b</sup>	
		mean	range	mean	range
Normal epidermis	19	3.0	(1.3- 5.0)	7.5	( 3.3-12.5)
3 paintings with benzene	6	2.3	(1.2- 2.9)	5.8	( 3.0- 7.3)
3 paintings with MC <sup>a</sup>	6	2.5	(2.0- 3.1)	7.4	( 5.9- 9.1)
6 paintings with MC	1	3.1	—	9.1	—
12 paintings with MC	7	2.8	(1.3- 3.7)	8.2	( 3.8-10.9)
18 paintings with MC	1	2.8	—	8.2	—
24 paintings with Me	1	3.3	—	9.7	—
Papillomata	6	3.6	(1.9- 5.6)	—	—
Carcinomata	19	9.0	(5.8-14.7)	48.9	(31.5-80.0)

<sup>a</sup> Methylcholanthrene.

<sup>b</sup> Dry weight values obtained from data in (14).

## DISCUSSION

It is interesting to summarize the changes observed in three enzyme systems studied to date during the process of epidermal carcinogenesis: cytochrome oxidase, succinic dehydrogenase, and apyrase. Throughout this discussion it should be kept in mind that what has been measured by the procedures employed is the potential, not effective, activity of the enzymes in the tissues studied (10). The numerous chemical and morphological changes that take place during epidermal carcinogenesis (13) may have important influences on the activity of the enzymes *in vivo*, but there is no way of evaluating these influences at the present time.

In the previous sections of this paper apyrase activity was expressed in terms of  $\gamma$  of P liberated/mg. of tissue (dry or wet weight)/15 min. In order to permit a direct comparison of the activity of apyrase with that of the other enzymes it is more convenient to express the activity

in terms of  $Q_p$ , *i.e.*, the quantity of P, expressed as  $\mu\text{l.}$  of gas, liberated/mg. of tissue/hr. (5). The results are expressed both on a wet and dry weight basis since there is no *a priori* reason for assigning greater validity to either method. It is probable that more nearly equal volumes of normal and tumor tissue are compared when wet weights are employed, since a given fresh weight of tumor tissue contains only approximately one-half of the dry weight of the same fresh weight of normal epidermis.

The results for both methods of calculation are shown in Fig. 7. When the results are expressed on the basis of wet weight the following changes are noted. The cytochrome oxidase activity remained virtually constant through 12 paintings, increased to 154 and 168% of the normal value after 18 and 24 paintings, respectively, and decreased to approximately 76% of normal in the carcinoma. Succinic dehydrogenase remained constant in the precancerous stages, but increased to 200% of normal in the carcinomata. No significance is attached to the slight fluctuations in apyrase activity prior to the tumor stage, at which time the activity was 3 times that of the normal epidermis.

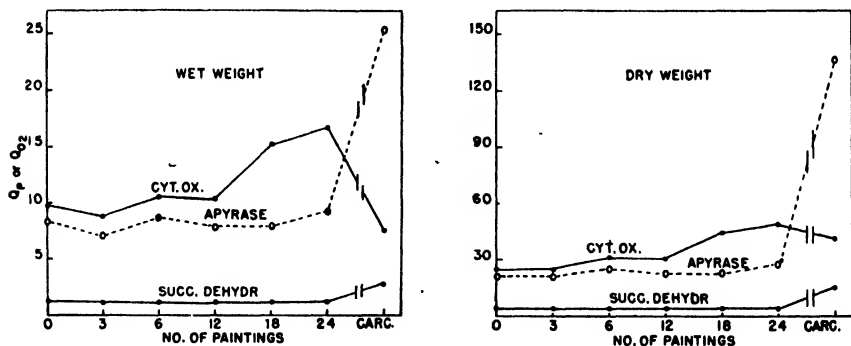


FIG. 7. Cytochrome oxidase, succinic dehydrogenase, and apyrase activities in epidermal carcinogenesis.

Thus, the succinic dehydrogenase and apyrase systems are similar to each other in their behavior and different from the cytochrome oxidase. The greatest relative increase in activity was shown by the apyrase system. When the results were calculated in terms of dry weight the cytochrome oxidase activity exhibited a rise not only during the late precarcinogenic hyperplasia, but also the tumors showed 168% of

normal activity. It is evident that whether it is concluded that there is an increase or decrease in cytochrome oxidase in these carcinomata as compared to normal epidermis depends on the mode of expression of the results. Succinic dehydrogenase and apyrase activities in the tumors were higher than normal, regardless of the manner of calculation, being 4.5 and 6.5 times, respectively, that of normal on a dry weight basis.

No great significance can be attached to the absolute values of the individual enzyme activities studied because of the uncertainty of the correct method for expressing the results. The relative changes of the enzyme activities are of greater interest since the *ratios* of the Q-values of the enzyme activities are the same for dry and wet weight, being independent of the basis of reference. The ratios of the Q-values for cytochrome oxidase, apyrase, and succinic dehydrogenase, respectively, are 1:0.85:0.15 for normal epidermis and 1:3.3:0.39 for tumors. Thus, both succinic dehydrogenase and apyrase increased in the carcinoma in comparison to the cytochrome oxidase, with the relative increase in apyrase activity being greater than that of the succinic dehydrogenase. The only other tissues for which comparable data are available are normal liver and hepatoma (11). The ratios of the Q-values for these same three enzymes, calculated as above, are for normal liver 1:0.4:0.38 and for hepatoma 1:1.2:0.27. In this case there is an increase in apyrase relative to cytochrome oxidase activity but little change in the relationship of succinic dehydrogenase to cytochrome oxidase. Thus, squamous cell carcinoma and hepatoma both possess a greater potential apyrase activity relative to the potential cytochrome oxidase and succinic dehydrogenase activities than do the normal parent tissues, epidermis and liver. If these findings have an actual correlative in cellular function then, perhaps, the accelerated glycolysis in tumors might in part be explained on the basis of an alteration in the balance between the adenylypyrophosphates resulting in an increased supply of adenylic phosphate acceptors coming from an increased apyrase activity inadequately compensated for by oxidative phosphorylations. Meyerhof (26) has demonstrated that the fermentation of hexosediphosphate by cell-free yeast preparations runs parallel to the distribution of apyrase activity when no glucose is present to serve as phosphate acceptor from ATP. In such preparations the rate of fermentation was found to be in close agreement with the apyrase activity. Glycolysis in intact tissues may possibly be regulated in a similar manner.



Although glycolysis and the metabolism of the adenylypyrophosphates are unquestionably connected with protein synthesis, the exact nature of the relationships is not clear because little is known of the actual mechanisms of regulation of these processes in the cell. It is not possible to attack directly the core of the cancer problem, which is one of an abnormality in protein synthesis, until the detailed knowledge of these mechanisms in normal cells is available.

### SUMMARY

1. There are enzymes present in normal and hyperplastic epidermis of mice and in the transplantable squamous cell carcinomata derived therefrom which catalyze the complete dephosphorylation of ATP.

2. The activity is not attributable to alkaline phosphatase since glycerophosphate is not hydrolyzed under the conditions employed.

3. No significant deviations from normal in adenylypyrophosphatase activity occur in mouse epidermis during the precancerous stages of epidermal carcinogenesis.

4. The mean value for the adenylypyrophosphatase activity of tumors was 3 times that observed for normal epidermis on the basis of wet weight and approximately 6.5 times on a dry weight basis.

5. The findings are discussed in relation to the values reported previously for cytochrome oxidase and succinic dehydrogenase in epidermal carcinogenesis and are compared to results obtained for normal liver and hepatomas.

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# Need for an Unidentified Factor or Factors for Growth, Reproduction, and Lactation of Rats Fed Purified Rations<sup>1</sup>

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Received August 4, 1947

## INTRODUCTION

Investigations reported by Cunha (1) and Ross and co-workers (2) showed that a basal ration of corn, soybean oil meal, minerals, and 5% alfalfa meal was inadequate for normal growth, reproduction, and lactation in the albino rat. Spitzer and Phillips (3) reported the presence of an active factor or factors in alfalfa meal, 1-20 liver powder, acid-washed casein plus choline, or fish meal. Using the same ration as used in (1) they found that this factor or factors was required for reproduction and lactation in the rat.

Evidence for an unidentified nutrient (X) required for growth, reproduction and lactation in the rat has been presented by Cary and Hartman (4). It has been suggested by McIntire *et al.* (5) that, to achieve optimum growth in rats, one or more factors are needed which are deficient in currently used purified diets with crystalline vitamins. This deficiency was corrected with liver extract. Zucker and Zucker (6), reported that this factor(s), whether it is an impurity or an essential amino acid is associated with proteins of good quality. These unknown factors were found to be very low or absent in alcohol-extracted casein (7).

McGinnis *et al.* (8) showed an unidentified factor to be required for reproduction in hens fed a soybean meal ration. This factor was supplied by meat scraps or 0.2% of Wilson and Company's "Liver fraction C." Later, this same liver preparation was shown to contain some factor which aided in the growth of chicks (9).

## EXPERIMENTAL

This work was conducted to study the supplemental effect on growth, reproduction and lactation in the rat of folic acid, biotin, inositol, *p*-aminobenzoic acid, a 95%

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<sup>1</sup>Published as Scientific Paper No. 727, College of Agriculture and Agricultural Experiment Stations, Institute of Agricultural Sciences, State College of Washington Pullman. This report is from a Thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Master of Science.

alcohol-soluble liver fraction,<sup>2</sup> or 15% of dehydrated alfalfa (cut at a height of 14 inches) containing 19.9% of protein, when added to a purified basal ration of 26.1% alcohol-extracted casein, 57.7% sucrose, 11.0% lard, and 5.2% minerals.<sup>3</sup>

To each kg. of this basal ration was added thiamine, 2 mg.; riboflavin, 3 mg.; choline, 1 g.; pyridoxine, 2 mg.; pantothenic acid, 6 mg.; niacin, 2.5 mg.; vitamin K, 5 mg.; and vitamin C, 25 mg. Vitamins A and D were fed orally in the form of oleum percomorphum at the rate of 1 drop/week/rat during growth and 2 drops/week during gestation and lactation.  $\alpha$ -Tocopherol was fed at the rate of 1 mg./week/rat. When alfalfa, containing 19.9% protein, was supplemented, the casein was lowered to 23.1% to maintain a constant protein intake. The rest of the alfalfa was added at the expense of the sucrose. When the liver fraction was supplemented, it was fed at 1.0% of the ration.

Females of Sprague-Dawley breeding were used. They were mated at 10-13 weeks of age. In the first and second trials the females were carried through growth as well as gestation and lactation on the purified diet, while in the third trial they were started on the experimental diet immediately prior to breeding. Two days after parturition all surviving litters were reduced to 6 young.

## RESULTS AND DISCUSSION

The results obtained during a six weeks growth period are summarized in Table I.

TABLE I  
*The Effect on Growth of Supplementing the Purified Basal Ration<sup>a</sup>*

Trail	Supplement to basal ration	No. of rats	Average weekly gains (6 wks.)	Average daily feed consumption	G. gain/g. feed
1	None	5	14.4	12.98	.158
	1% Liver paste	5	12.1	11.36	.152
	Folic acid	5	8.9	14.09	.090
	4 B-vitamins	5	14.3	12.67	.161
2	None	6	8.9	9.93	.128
	15% Dehydrated alfalfa	6	20.6	13.33	.221

<sup>a</sup> Rates of supplementation were folic acid, 6  $\gamma$ /day; biotin, 1  $\gamma$ /day; *p*-aminobenzoic acid, 500 mg./kg. of feed; inositol, 3 g./kg. of feed.

<sup>2</sup> The liver fraction was the same as that used by McGinnis and co-workers (8, 9) and which was shown by these authors to contain an unidentified factor or factors for the chicken.

<sup>3</sup> The purified basal ration was originally used for swine by Wintrobe (10).

From the data in Table I it may be seen that the addition of the liver fraction, or of the 4 B-vitamins, biotin, inositol, *p*-aminobenzoic acid and folic acid had no supplemental effect on growth. There was considerable depression of growth by the addition of folic acid alone. This effect of folic acid might be due to a vitamin imbalance since Cunha *et al.* (11, 12) found that the addition of one vitamin of the B-complex without others accentuated poor growth in the rat and poor reproduction in the sow, respectively. In these experiments, it appears that there was no need for biotin, inositol, *p*-aminobenzoic acid, or folic acid in the ration of the rat for growth when the other 6 B-complex vitamins were supplied in the ration.

As growth was not optimal, the rat must require one or more unknown factors for growth over and above the ten known B-complex vitamins. This is in agreement with the recent findings of Sporn, Ruegamer and Elvehjem (13). The liver fraction which contained an unknown factor(s) for the chick, gave no supplemental effect so this would probably suggest that a different unknown factor(s) is concerned in the growth of the chick than in the growth of the rat. However, it may be that the factor(s) differs in its availability to these two species. The addition of 15% alfalfa to the basal ration more than doubled growth over its control. This large supplemental effect given by alfalfa is in agreement with work of Cunha (1) and Spitzer and Phillips (3) where alfalfa was shown to contain some factor or factors needed to supplement a natural ration.

Table II summarizes the performance of 62 females during reproduction and lactation.

The results shown in Table II clearly indicate that the basal ration was not satisfactory for reproduction and lactation in the rat. Ensinger, Bowland and Cunha (14) reported that, on an identical basal ration, there was a deficiency of one (or more) unknown factor(s) for reproduction and lactation in the sow.

The conception rate was not good in the rats fed the basal diet and was not improved by supplements of liver paste or folic acid alone. The addition of the 4 B-complex vitamins or alfalfa gave considerable response in rate of conceptions. All the supplements were significantly helpful in increasing gains during gestation, but alfalfa gave the greatest response. The addition of folic acid alone or of the 4 B-complex vitamins increased the liveability of the young and was, therefore, considered as improving the ability of the females to lactate. Fifteen

per cent alfalfa gave a large response in increased liveability of the young and improved lactation of the females.

An average of 9% of the rats in all lots, except the alfalfa supplemented lot, had a type of hemorrhage of the fetal attachments which resulted in expulsion of the dead fetus at term, partial resorption, or toxemia and death of the female. A similar condition has been reported by Cunha (1) and Spitzer and Phillips (3) in rats fed natural rations.

TABLE II  
*Effect on Reproduction and Lactation of Supplementing  
the Purified Basal Ration<sup>a</sup>*

Supplement to basal ration <sup>b</sup>	Number of females	Number of litters	Possible conceptions which occurred with females bred	Average weight gains during gestation	Average number living rats born per litter	Number of litters weaned	Average length of time young rats survived	Per cent weaned of young given to females to raise
			<i>per cent</i>	<i>g.</i>			<i>days</i>	<i>per cent</i>
None	17	16	72.7	30.4	5.37	0	1.81	0
1% Liver paste	11	12	75.0	58.1	6.42	0	1.36	0
Folic acid	11	11	68.7	62.4	8.09	1	3.83	7.6
4 B-vitamins	11	15	93.7	63.2	7.53	2	4.43	13.0
15% Dehydrated alfalfa	12	11	91.7	75.3	6.36	5	10.12	43.7

<sup>a</sup> Rates of supplementation the same as during growth except that biotin was fed at a rate of 5  $\gamma$ /day.

<sup>b</sup> These data include the results of three trials. The first and second trials were those for which data on growth were obtained. The third trial was a repetition of the first two but data on reproduction only were obtained. Since the results of all trials were similar, the data obtained were combined in this table.

The addition of folic acid to the basal ration gave some supplemental effect on gestation gains and lactation. Biotin, inositol, *p*-aminobenzoic acid, and folic acid when fed together gave a similar improvement in gestation gains and lactation and also an increase in conceptions. It would therefore appear that folic acid and one or all of the other three B-vitamins (biotin, inositol and *p*-aminobenzoic acid) were of some benefit to the rat for reproduction and lactation.

It appears that, in addition to the ten known B-complex vitamins, the rat requires one or more unrecognized factors for reproduction and lactation. This confirms the recent finding of Sporn, Ruegamer and

Elvehjem (13) where liver contained some factor(s) which increased survival in the young. This may be the same or some different factor or factors to that required for growth. The same liver paste, which contained an unknown factor(s) for hatchability in the hen, had no supplemental effect so this would suggest that a different factor or factors is concerned in reproduction in hens and rats. However, it may be that the availability of this factor(s) differs between the rat and the hen. When fed at a level of 15% of the ration, dehydrated alfalfa gave a large supplemental effect in reproduction and lactation of the females and liveability of the young. However, the ability of the females to wean their young was still not normal, thus indicating that the 15% level of alfalfa used in these trials did not contain all or enough of the unknown factor or factors needed to maintain satisfactory lactation.

#### SUMMARY

A purified diet containing 26.1% alcohol-extracted casein; 57.7% sucrose; 11.0% lard; 5.2% minerals; vitamins A, D, E, K, C; and the 6 B-complex vitamins, thiamine, riboflavin, choline, nicotinic acid, pantothenic acid, and pyridoxine, gave only fair growth, poor reproduction, and complete failure of lactation in the albino rat.

The addition of folic acid alone to the basal ration was deleterious to growth but of some aid in reproduction and lactation. Supplements of biotin, inositol, *p*-aminobenzoic acid and folic acid together did not aid growth but improved reproduction and lactation.

A factor or factors other than the 10 B-complex vitamins is apparently required for optimum growth and for satisfactory reproduction and lactation in the rat. Alfalfa is a source of these factors, as the addition of a level of 15% of dehydrated alfalfa in the ration increased growth to a great extent, gave normal reproduction, and improved lactation considerably. A 95% alcohol-soluble liver fraction which contains an unknown factor or factors for hatchability in the hen and for growth in the chick was of no benefit to the rat for growth, reproduction, and lactation when added to the ration at a level of 1%.

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# Fatty Acid Transformations by Anaerobic Bacteria <sup>1</sup>

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## INTRODUCTION

There is little factual information relating to the means by which fatty acids are transformed anaerobically in nature. The bacterial consumption or alteration of many fatty acids has been observed repeatedly under aerobic conditions. Acids involved have varied considerably in chain length and have included both saturated and unsaturated types (2, 4, 6, 10, 11, 12, 13, 15, 20). The rates of aerobic change fluctuate among species and substrates, but Peppler (17) has reported certain fatty acids to be more readily oxidized than glycerol by *Pseudomonas aeruginosa*.

The claim that fatty acids are not subject to decomposition by anaerobic bacteria (19) has been successfully challenged by several investigators. Clarke and Mazur (5) found that the rate of disappearance of fatty acids from diatoms and marine muds was greater under nitrogen than in an aerobic environment. The range of fatty acids consumed anaerobically is apparently as great as that attacked under aerobic conditions (27). The anaerobic nature of the bacteria involved is indisputable, for included are the genera *Clostridium* and *Desulfovibrio* (1, 7, 16, 18).

Anaerobic oxidation of fatty acids has been observed. Stone *et al.* (25, 26) noted that various species of *Propionibacterium* activated formic, acetic, and propionic acids as hydrogen donors. In these reactions methylene blue, nitrates, or *o*-chlorophenolindophenol functioned as hydrogen acceptors. Formic acid also served as an oxidant in a few instances. The dehydrogenation of formic acid has been activated also by anaerobic lipoclasts (21). Peculiarities in the behavior of formic acid have tended to exclude it from classification as a fatty acid. Nevertheless, its importance in the anaerobic changes affecting fatty acids is sufficiently great to warrant its inclusion in any such study.

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<sup>1</sup> Contributions from the Scripps Institution of Oceanography, New Series No. 357. This paper is a contribution from American Petroleum Institute Research Project 43A.

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Some type of dehydrogenation may account for the observations of Reynolds and Hopkins (20), who reported increases in the iodine number of oleic acid subjected to bacterial attack. However, the desaturating effect was greater under aerobic than anaerobic conditions.

In some cases bacteria have induced hydrogenation and, possibly, other types of fatty acid reduction. One such reducing influence is that affecting unsaturated acids. Schönbrunner (23) repeatedly observed saturation of oleic and sorbic acids by various soil and mud samples as well as by several pure bacterial cultures. The acids were saturated to more than 60% of completion. Saturation of lipides by bacteria in an anaerobic environment also is reported by Ginsburg-Karagicheva and associates (8, 9).

Bacteria are likewise known to reduce saturated acids. Prominent among these compounds are the volatile fatty acids important as fermentation intermediates. The data of Blanchard and MacDonald (3), Mickelson and Werkman (14), and Severson (24) illustrate the reduction of propionic and butyric acids to their corresponding alcohols. The conversions evidently represent a direct reduction of the carboxyl group, for isomeric configurations were preserved in the transition from fatty acid to alcohol. Severson (24) showed that the transformation product of acetic acid was almost entirely acetone, which also represented a partial reduction product of propionic and butyric acids. It is significant that the production of gaseous hydrogen during fermentation was depressed as acetic and propionic acids were reduced (14). This demonstration provokes speculation concerning the possible activation of hydrogen and its consumption as an agent in the reduction of the carboxyl group.

The bacterial reduction of saturated fatty acids is not confined to water-soluble compounds. ZoBell (31) has described the alteration of capric acid under the influence of a mixed culture containing the strongly anaerobic sulfate-reducing bacteria. This culture produced an oil-like, ether-soluble, unsaponifiable material. Determinations of refractive index, birefringence, and optical activity tended to classify the material as a normal paraffin. Most of the crystals appeared to be  $C_{22}H_{44}$ , or in the range from  $C_{20}$  to  $C_{26}$ . The behavior of the crystals when heated was similar to that of the petroleum ceresins. The mechanism of the transformation was not determined and may have included decarboxylation and polymerization reactions. There have been several other reports of the bacterial reduction of fatty acids to petroleum hydrocarbons, but few of them are more than unsubstantiated speculations.

These isolated descriptions of the bacterial utilization of fatty acids coupled with the demonstration of lipide hydrolyses by anaerobic bacteria (22) have emphasized the importance of tracing the further transformation or degradation of the liberated fatty acids. The following data may be of some aid in this endeavor.

#### TRANSFORMATION OF FATTY ACIDS BURIED IN MARINE SEDIMENTS

Examination of marine sediments affords proof of the anaerobic changes affecting naturally occurring fatty acids. Results given in

Table I describe lipid fractions of marine mud core SL601 and represent an average of duplicate determinations.

TABLE I  
*Vertical Distribution of Lipides in Marine Sediments*

Lipide material	Core level in inches	
	14-20	40-48
Estimated age, in years	210-300	600-720
Unsaponifiable material, per cent by dry weight	0.0115	0.0076
Fatty acids, per cent by dry weight	0.0085	0.0052
Molecular weight	355	383
Iodine number	52	26

Two levels of the core were dried at 70°C., powdered, and extracted in a Soxhlet apparatus with alcohol-ether (3:1). The crude extract was fractionated according to the gravimetric procedure of Wilson and Hansen (29), and the iodine numbers were determined by the method of Yasuda (30).

Quantitative distribution of the lipides is not susceptible to exact interpretation. The amounts recovered depend upon several factors, including stratification at deposition and bacterial consumption or production. However, the characteristics of the extracted fatty acids are of more interest. Increases, with geologic age, in acid chain-length can perhaps be accounted for on a basis of preferential consumption of shorter molecules and the possible bacterial alteration of existing compounds. Similar reasoning may be applied to the saturation of fatty acids with depth. The possibility that stratification was responsible for these important differences in the degrees of saturation was minimized by studies on additional material. Analyses are summarized in Table II.

TABLE II  
*Saturation Gradient of Fatty Acids in Marine Sediments*

Lipide material	Core level in inches					
	0-8	8-18	18-24	30-36	36-40	40-44
Estimated age, in years	0-80	80-180	180-240	300-360	360-400	400-440
Fatty acids,						
Per cent by dry weight	0.0179	0.0241	0.0110	0.0143	0.0306	0.0139
Iodine number	45	50	33	35	30	27

Determinations were similar to those described above, except that the core sections were dried under conditions which minimized autooxidation of labile material. Im-

mediately upon collection at sea, the core (XXIX:149-102) was sectioned and placed in glass jars, each of which was then completely filled with sea water and sealed. Oxygen dissolved in the water was reduced quite rapidly, for a pronounced odor of hydrogen sulfide was noted when the jars were opened for subsequent treatment. Within 3 hours of collection the muds were transferred to acetone, sealed, and stored at room temperature for 24 hours. Then followed similar treatment with several charges of absolute ethyl alcohol. Desiccation was completed by *in vacuo* treatment over Drierite at room temperature. The acetone-alcohol filtrates were pooled and combined with subsequent alcohol-ether extracts. These combinations were reduced in volume by vacuum distillation prior to fractionation.

The quantitative distribution of fatty acids again appears to be without significance, but the changes in iodine number are important. Although exceptions to a smooth curve are evident, there is ample evidence of a general saturation with increasing age of the acid.

The data do not indicate whether the saturations were the result of a preferential consumption of unsaturated fatty acids, a reduction at double bonds, or a combination of both factors. Further, the changes in the fatty acid content of sediments are not in themselves direct evidence for the activity of anaerobes, although they strongly suggest the functioning of bacterial agents. Determination of the motivation for these, and other, changes in naturally occurring fatty acids prompted more direct studies into the effects of anaerobic bacteria upon such compounds.

#### GROWTH OF ANAEROBES UPON FATTY ACIDS

Both facultative and obligate anaerobes are known to hydrolyze lipides in anaerobic environments. The two types of bacteria were, therefore, tested for their abilities to utilize fatty acids as sources of carbon and energy. Table III depicts the results observed.

Cultures were prepared in glass-stoppered bottles, employing the mineral salts solution and techniques described previously (22). Fatty acids were added as potassium salts and in amounts sufficient to give a final concentration of 0.05%. Inocula were obtained from several types of mud and water samples which harbored large numbers of facultative and obligate anaerobes capable of lipid hydrolysis. Duplicate series of cultures were included and contained 0.05% of glycerol in addition to the fatty acids as a check upon inoculum viability and fatty acid toxicity. Cultures were examined for growth during incubation for 34 days at 27°C.

Culture CS-1, the only inoculum to develop consistently upon fatty acids without glycerol, was able to utilize both saturated and unsaturated acids. Its failure to grow upon caprylic and pelargonic

TABLE III

*Growth of Anaerobes on Fatty Acids and on Fatty Acid-Glycerol  
Combinations During Incubation for 34 Days at 27°C.*

Substrate	Growth of inoculum					
	D-1	PE-2	CS-1	SS-1	SL490-1	M-1
Mineral salts solution	—	—	—	—	—	—
Glycerol	+	+	+	+	+	+
Saturated:						
Formic acid	—	—	+	+	—	—
Formic acid + glycerol	—	—	+	+	—	—
Acetic acid	—	—	+	—	—	—
Acetic acid + glycerol	—	—	+	+	—	—
Propionic acid	—	—	+	—	—	—
Propionic acid + glycerol	—	—	+	—	—	—
Butyric acid	—	—	+	—	—	—
Butyric acid + glycerol	—	—	+	—	—	—
Valeric acid	—	—	+	—	—	—
Valeric acid + glycerol	—	—	+	—	—	—
Caproic acid	—	—	+	—	—	—
Caproic acid + glycerol	—	—	+	—	—	—
Enanthic acid	—	—	+	—	—	—
Enanthic acid + glycerol	—	—	+	—	—	—
Caprylic acid	—	—	—	—	—	—
Caprylic acid + glycerol	—	—	+	—	—	—
Pelargonic acid	—	—	—	—	—	—
Pelargonic acid + glycerol	—	—	—	—	—	—
Capric acid	—	—	+	—	—	—
Capric acid + glycerol	—	—	+	—	—	—
Lauric acid	—	—	+	—	—	—
Lauric acid + glycerol	—	—	+	—	—	—
Myristic acid	—	—	+	—	—	—
Myristic acid + glycerol	—	—	+	—	—	—
Palmitic acid	—	—	+	—	—	—
Palmitic acid + glycerol	—	—	+	—	—	—
Stearic acid	—	—	—	—	—	—
Stearic acid + glycerol	—	—	+	—	—	—
Unsaturated:						
Undecylenic acid	—	—	+	—	—	—
Undecylenic acid + glycerol	—	—	+	+	—	—
Oleic acid	—	—	+	—	—	—
Oleic acid + glycerol	—	—	+	+	—	—

acids is probably attributable to the toxicity of their soluble salts. Potassium stearate was converted to highly insoluble calcium and magnesium salts apparently incapable of supporting growth. It should be noted that only inoculum CS-1 produced a sulfate-reducing culture which, of course, was obligately anaerobic. Fatty acids failed to support the growth of facultative cultures, proved to be viable by their development upon glycerol. This circumstance may be related to the lack of a reducible substance, although growth failure upon combinations of acids and glycerol suggests that substrate toxicity was paramount.

A large group of mixed cultures, each containing sulfate-reducing anaerobes, was next tested upon a series of fatty acids added as potassium salts in the manner described above. The cultures were examined during a period of 30 days for evidence of sulfide production and, thus, fatty acid oxidation. The distribution of fatty acid-oxidizing cultures is noted in Table IV.

TABLE IV  
*Growth of Sulfate-Reducing Cultures on Fatty Acids  
During Incubation for 30 Days at 27°C.*

Acid	Cultures tested	Cultures which developed
Acetic	62	14
Propionic	62	4
Butyric	62	4
Valeric	62	3
Caproic	62	3
Enanthic	62	3
Caprylic	62	1
Pelargonic	62	3
Capric	62	3
Lauric	62	4
Myristic	62	4

The ability of sulfate reducers to oxidize fatty acids appears to be limited by two factors, toxicity and solubility of substrate. The cultures were tested in a sea-water medium, and a considerable portion of the substrate was precipitated in the form of calcium and magnesium salts. The precipitation was most pronounced with acids above the caprylic level. Thus, while the inhibitory powers of myristate for sulfate reducers may be greater than those of caprylate, the greater solubility of the latter results in more pronounced toxic effects.

## FATTY ACID DEHYDROGENATIONS

The indicated ability of sulfate reducers to oxidize a variety of fatty acids, and the failure of non-sulfate-reducing anaerobes to do so, led to an investigation of these behaviors by other than cultural means.

Cultures were purified prior to enzymatic studies. The incidence of fatty acid dehydrogenations is described in Table V.

TABLE V  
*Dehydrogenation of Fatty Acids by Anaerobic Bacteria*

Acid	Non-sulfate reducers		Sulfate reducers	
	Tested	Active	Tested	Active
Saturated:				
Formic	7	7	5	2
Acetic	7	1	5	2
Propionic	7	1	5	0
Butyric	7	3	5	2
Valeric	7	2	5	1
Caproic	7	2	5	1
Enanthic	7	1	5	0
Caprylic	6	0	5	1
Capric	6	0	5	0
Lauric	6	1	5	0
Myristic	6	0	5	0
Palmitic	6	1	5	1
Stearic	6	2	5	4
Unsaturated:				
Undecylenic	6	0	5	0
Oleic	6	1	5	1
Linoleic	5	3	5	2
Linolenic	5	3	5	2

Cells were prepared by growing non-sulfate-reducing cultures in 5-gal. bottles filled with a mineral salts solution supplemented by 1.0% of Bacto peptone, 0.3% of yeast extract, and 0.02% of ascorbic acid. The filled and inoculated bottles were fitted aseptically with a sterile rubber stopper carrying a mercury check-valve, although free gases were not generally produced. The growth period was 24-48 hours at 27°C. Sulfate reducers were cultivated similarly, except that 1.0% of glucose was added. Cultures were incubated for 48-72 hours at 27°C. Cells were recovered by passage of the culture through a supercentrifuge. The cellular crop was washed 3 times in sea water and resuspended in the same medium. Dehydrogenase activities were



estimated by the Thunberg method as modified by Umbreit *et al.* (28). Endogenous oxidation customarily reduced methylene blue in from 30 to 60 minutes, and fatty acid dehydrogenation was indicated by a decrease in the time required for decoloration of the dye when a substrate was added.

Noticeable is the predominant ability of non-sulfate-reducing lipoclasts to dehydrogenate formic acid, none of the cultures failing in this respect. The failures in activation of acids between the heptylic and lauric levels may be due to the toxicity of their salts. Theoretically, the toxic effects should continue to increase with carbon chain length, but the insolubility of salts of the higher acids is believed to have reduced their effective concentrations to subinhibitory levels, and thus to have permitted their dehydrogenation.

The range of fatty acids subject to dehydrogenation by sulfate reducers is somewhat similar to that described above. A difference is observed, however, in the affinity of non-sulfate-reducing lipoclasts for formic acid as contrasted with that of the sulfate reducers for stearic acid. There are the same general limitations to the dehydrogenation of intermediate fatty acids and presumably for the same reasons.

#### HYDROGEN TRANSFER IN THE FORMIC ACID-LINOLEIC ACID SYSTEM

The ready dehydrogenation of formic acid suggested that this compound might, upon activation, provide a source of hydrogen for the saturation of other organic substances. Accordingly, a test system was prepared employing linoleic acid as a potential hydrogen acceptor. Data given in Table VI illustrate the progress of the reactions.

Reactants were added to 125 ml. Erlenmeyer flasks, each of which received 10 ml. of *M*/15 phosphate buffer, pH 7.2, and 5 ml. of washed cell suspension. Formic acid, *M*/100, and linoleic acid, *M*/200, were added in 10 ml. quantities and as potassium salts. Distilled water was added, when necessary, to maintain identical volumes in all flasks. The mixtures were prepared in duplicate and incubated *in vacuo* for 24 hours at 27°C. They were then acidified with HCl and extracted with petroleum ether, the solvent being subsequently evaporated under reduced pressure at room temperature. To avoid prolonged treatment in air, no attempt was made to extract the preparations to completion. The iodine number was determined by the method of Yasuda (30). Although the extracts represent both linoleic acid and cellular lipides, the bulk of the material was obtained from the linoleic acid added. Thus, the iodine number is largely a reflection of the degree of saturation of this acid.

TABLE VI  
*Hydrogenation of Linoleic Acid by Anaerobic Bacteria*

Culture	Acid	Extract	
		Grams	Iodine number
XXIX:129-2 (non-sulfate reducer)	Formic	0.0019	5.3
		.0016	4.8
	Linoleic	0.0127	76.2
		.0156	79.3
	Formic + Linoleic	0.0269	26.4
		.0125	29.0
XXIX:136 9 (sulfate reducer)	Formic	0.0011	7.1
		.0021	6.5
	Linoleic	0.0207	81.1
		.0200	77.5
	Formic + Linoleic	0.0230	23.0
		.0271	26.2
XXIX:137-1 (non-sulfate reducer)	Formic	0.0030	7.0
		.0022	7.4
	Linoleic	0.0304	80.7
		.0316	83.1
	Formic + Linoleic	0.0340	22.0
		.0355	19.7
XXIX:137-12 (non-sulfate reducer)	Formic	0.0060	6.4
		.0071	5.5
	Linoleic	0.0324	83.1
		.0346	80.6
	Formic + Linoleic	0.0371	23.0
		.0380	21.6

The hydrogenation of linoleic acid in these redox systems is obvious. Of the four cultures employed, two were known to be capable of dehydrogenating linoleic acid in addition to formic acid, and two were not. Possession of a linoleic acid dehydrogenase is, however, apparently quite unrelated to the ability to saturate the acid, since reduction proceeded with remarkable similarity in all cases.

### SUMMARY

Data presented give evidence that fatty acids of the types preformed in nature or released by the hydrolysis of esters can undergo further alteration through the agency of anaerobic bacteria. Organisms tested have been of two general groups: obligately anaerobic sulfate-reducing *Desulfovibrio* and species of facultatively anaerobic lipoclasts which did not reduce sulfates.

The transformation of naturally occurring fatty acids in marine sediments was traced to a limited extent and attributed to the presumable activity of anaerobic bacteria. Saturation of fatty acids buried in sedimentary materials was observed to increase with depth, *i.e.*, with geologic age.

Fatty acids were subject to oxidation by anaerobes. No information was obtained concerning the specificity of fatty acid dehydrogenases, but the ability to dehydrogenate the acids was quite generalized among the anaerobes investigated. Among the important factors affecting dehydrogenation are toxicity and solubility of the substrate.

The pronounced reducing effects of bacterially activated formic acid were sufficient to induce considerable hydrogenation of linoleic acid.

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# The Structure of the Alkaloid of YOLOXOCHITL

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Received August 8, 1927

## INTRODUCTION

Yoloxochitl, the "flower of the heart," is a plant belonging to the *Embriophyta sifonogama* division, subdivision Angiosperm, class Dicotyledonea, subclass Arquiclamidea, order of the Ranales, family Magnoliaceae, genus *Talauma*, species Mexican.

This plant was used by the Indians many years ago as a heart stimulant (1). The subject of this paper is the isolation of the alkaloid from this plant and the determination of its structural formula.

## EXPERIMENTAL

### *Isolation of the Alkaloid*

Five kg. of dry powdered Yoloxochitl leaves were moistened with a 10% ammonium hydroxide solution and covered with ethylene chloride. The extraction lasted a week, after which the mass was filtered, first through gauze and then through paper. The filtrate was mixed mechanically with an equal volume of 10% sulphuric acid and allowed to settle, when two layers stratified. By decanting, the sulphuric acid layer was removed. This was filtered and neutralized with ammonium hydroxide, and dehydrated *in vacuo*. The residue was dried in a vacuum drier with phosphorus pentoxide, later moistened with ammonium hydroxide, and the alkaloid extracted with ethylene chloride. The solvent was removed *in vacuo* and the crude alkaloid obtained. This crude product was boiled with benzene, filtered, dried, and crystallized from ethylene chloride until the fusion point became constant at 176°C. The yield was 225 mg. This extraction was repeated 6 times using a total of 30 kg. of dry leaf powder. Analysis: C, 70.3; H, 6.4; N, 4.4. The simplest possible formula thus seems to be:  $C_{38}H_{40}O_7N_2$ .

### *Determination of the Methoxy Groups*

One-tenth g. of the alkaloid was treated with 6 cc. of hydriodic acid (density 1.7) and heated, the methyl iodide formed was blown out with carbon monoxide and bubbled through concentrated alcoholic silver nitrate solution, after running the gaseous

mixture through red phosphorus. The  $\text{AgI} \cdot 2\text{AgNO}_3$  was precipitated, filtered, washed with water, and the insoluble silver iodide weighed. This weight was 76 mg. corresponding stoichiometrically to 2 methoxy groups.

### *Methylation of the Alkaloid and Determination of the Hydroxyl Groups*

Another 0.1 g. of the alkaloid was methylated according to the Cornforth (2) technique using methanolic sodium methoxide. The product was purified with benzene and ethyl chloride and the number of methoxy groups determined.

Silver iodide = 229 mg.,

corresponding to 6 methoxy groups. Subtracting the two previously found, we obtain 4 hydroxyl groups corresponding to the 4 extra methoxy groups.

### *Confirmation of the Number of Hydroxyl Groups*

For this purpose 10 mg. of the alkaloid were reacted with methyl magnesium iodide according to the Zerewitinoff (3) method, using a microburette for the measurement of the methane.

The methane found and recovered was 1.38 cc., corresponding to 4 hydroxyl groups.

### *Determination of the $>\text{N}-\text{CH}_3$ Group*

The method employed was that of Feigl, Anger and Zappert (4), using fluorescein chloride and anhydrous zinc chloride with 10 mg. of the compound. A blue color developed, indicating the presence of  $-\text{NH}_2$ ,  $>\text{NH}$ , or  $>\text{N}-\text{CH}_3$  groups.

To another 10 mg. the Hinsberg (5) reaction was applied to distinguish between primary, secondary, and tertiary amines. The test with benzenesulphonyl chloride indicated the presence of a tertiary amine.

### *Splitting of the Alkaloid at its Ether Group*

The alkaloid (500 mg.) was treated with hydriodic acid, using the Remick method (6). The ether is split by hydriodic acid. The residue was fractionated with ether, two fractions being obtained:  $\text{C}_{18}\text{H}_{20}\text{O}_3\text{NI}$  and  $\text{C}_{18}\text{H}_{21}\text{O}_4\text{N}$ . Anal.: for  $\text{C}_{18}\text{H}_{20}\text{O}_3\text{NI}$ , calc., C, 50.8; H, 4.7; N, 3.3; I, 29.9; found, C, 50.6; H, 4.4; N, 3.1; I, 29.5. For iodine the Riggs, Lavietes and Man (7) technique was followed. Anal.: for  $\text{C}_{18}\text{H}_{21}\text{O}_4\text{N}$ , calc., C, 68.5; H, 6.6; N, 4.4; found, C, 68.2; H, 6.5; N, 4.1.

### *Determination of the $-\text{CH}_2-$ Group in $\text{C}_{18}\text{H}_{20}\text{O}_3\text{NI}$ and $\text{C}_{18}\text{H}_{21}\text{O}_4\text{N}$*

The reactions with sodium 1,2-naphthaquinone-4-sulfonate in 50% alcohol (8) were made on small quantities of the above compounds with a positive result, i.e., a red color, denoting that the  $\text{C}_{18}\text{H}_{20}\text{O}_3\text{NI}$  and  $\text{C}_{18}\text{H}_{21}\text{O}_4\text{N}$  contain the  $-\text{CH}_2-$  group.

*Methylation and Oxidation of the  $C_{18}H_{20}O_3NI$  and  $C_{18}H_{21}O_4N$  Fractions*

From each fraction, 0.1 g. was methylated by the above mentioned process; oxidation was then carried out using 1 g. of chromic anhydride solution in 4 cc. of water and 4 cc. of glacial acetic acid, mixing mechanically while cooling in an ice bath. The extraction of the crude products resulting from both experiments was made with ethyl chloride. By evaporation, the identification of 1-keto-2-methyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline was made in both instances with a melting point of 124°C. (literature: 125°C.). Anal.: N for  $C_{12}H_{16}O_3N$ , calc. 6.3; found, (1st fraction) 6.1; (2nd fraction) 6.1.

*Acetylation of the Alkaloid with Acetic Anhydride and Oxidation*

One hundred mg. of the compound were treated with 5 cc. of acetic anhydride and refluxed for an hour. The compound was then added dropwise during one hour to a solution of 1 g. of chromic anhydride in 4 cc. of water and 4 cc. of glacial acetic acid with mechanical stirring while cooling in an ice bath.

The final product was poured into water and boiled under a reflux. By extracting with ethyl chloride we could identify 1-keto-2-methyl-6-methoxy-7-hydroxy-1,2,3,4-tetrahydroisoquinoline (m.p. 119°C.). Anal.: N for  $C_{11}H_{13}O_3N$ , calc. 6.7; found 6.3.

*Determination of the Position of the Hydroxy and Ether Groups*

On 300 mg. of the alkaloid the former procedures for methylation, oxidation and rupture of ether were repeated. The crude mass was treated with an equal weight of silver oxide in water (9), and the final product extracted with ether, giving a substance which had a melting point of 205°C. and whose *p*-nitrobenzyl ester melted at 140°C., corresponding to vanillic acid.

## DISCUSSION

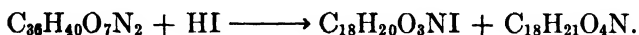
The alkaloid was obtained from leaves of yolochochitl by extraction with ethylene chloride. Its color is light brown and it has a melting point of 176°C. It is soluble in ethylene chloride, slightly soluble in chloroform, and insoluble in water, ether and alcohol. Its hydrochloride is freely soluble in water. It gives positive results with the Bouchardat, Mayer, Valser, Dragendorff-Yvon, Scheibler and Froehde tests. The compound was investigated by micro qualitative analytic procedures and found to contain C, H, and N, but no S or halogens. The following quantitative relation was found on micro analysis: C, 70.3; H, 6.4; N, 4.4. Thus, the empirical formula should be  $C_{36}H_{40}O_7N_2$ . This is corroborated by the molecular weight of the compound, determined by the Rast method. The calculated molecular weight is 612 and the value actually found is 608. The presence of two methoxy



groups was established by the weight of silver iodide formed when the compound was reacted with hydriodic acid and the gases bubbled through a solution of silver nitrate. The presence of four hydroxyl groups was established in the same manner; for this the free hydroxyl groups were methylated and the methoxy groups then determined. This test was confirmed by measuring the amount of methane given off when the compound was reacted with methyl magnesium iodide as per Zerewitinoff.

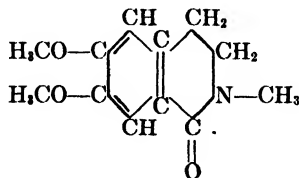
The nitrogen atoms in the alkaloid were found in the following manner.  $>N\cdot CH_3$ : the presence of this group was confirmed with fluoresceine chloride according to the method of Anger and Zappert, and by the Hinsberg technique was found to be a tertiary amine.

Since the compound has two methoxy and four hydroxyl groups and the total number of oxygen atoms is seven, the remaining oxygen is present as an ether, since the alkaloid did not react with 2,4-dinitrophenylhydrazine. The ether link was ruptured by Remick's method, and the compound broken down according to the following equation:



By micro analysis we found: C, 50.6; H, 4.4; N, 3.1; I, 29.5; which coincide with the theoretical values: C, 50.8; H, 4.7; N, 3.3; I, 29.9, for  $C_{18}H_{20}O_3NI$ . Likewise, the second compound gave the following values: C, 68.2; H, 6.5; N, 4.1; which coincide with theoretical values of: C, 68.5; H, 6.6; N, 4.4, for  $C_{18}H_{21}O_4N$ .

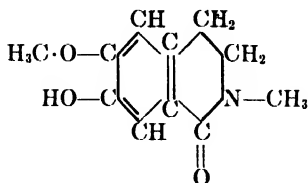
By the use of sodium 1,2-naphthaquinone-4-sulfonate we identified the presence of the  $-CH_2-$  group in both the  $C_{18}H_{20}O_3NI$  and  $C_{18}H_{21}O_4N$  fractions. The  $C_{18}H_{20}O_3NI$  and  $C_{18}H_{21}O_4N$  fractions were then methylated and later oxidized with chromic anhydride, after which 1-keto-2-methyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline was isolated.



This has a melting point of  $124^{\circ}C$ . in both cases. After this we proceeded to quantitative micro analysis. Calculated analysis for  $C_{12}H_{16}$ -

$O_3N:N$ , 6.3; found, N, 6.1 (in the first fraction); N, 6.1 (in the second fraction).

The product was acetylated with acetic anhydride and oxidized with a solution of chromic anhydride in acetic acid. Later, by hydrolysis, the acetyl groups were eliminated and, after extracting with ethyl chloride, we identified 1-keto-2-methyl-6-methoxy-7-hydroxy-1,2,3,4-tetrahydroisoquinoline, which has a melting point of  $119^\circ\text{C}$ . Calculated



lated analysis for  $C_{11}H_{13}O_3N$ : N, 6.7; found: N, 6.3. This indicates that the hydroxyl is on the seventh carbon of the isoquinoline, both in the  $C_{18}H_{20}O_3NI$  and  $C_{18}H_{21}O_4N$  fractions.

Finally, the alkaloid was methylated, oxidized and cracked at its ether linkage, when we obtained an iodized fraction in which the iodine was transformed to a hydroxyl by means of aqueous silver oxide; the aggregate was then extracted with ether. Vanillic acid was identified in two ways. First by its melting point,  $205^\circ\text{C}$ ., and secondly, by preparing its *p*-nitrobenzyl ester, which melted at  $140^\circ\text{C}$ .

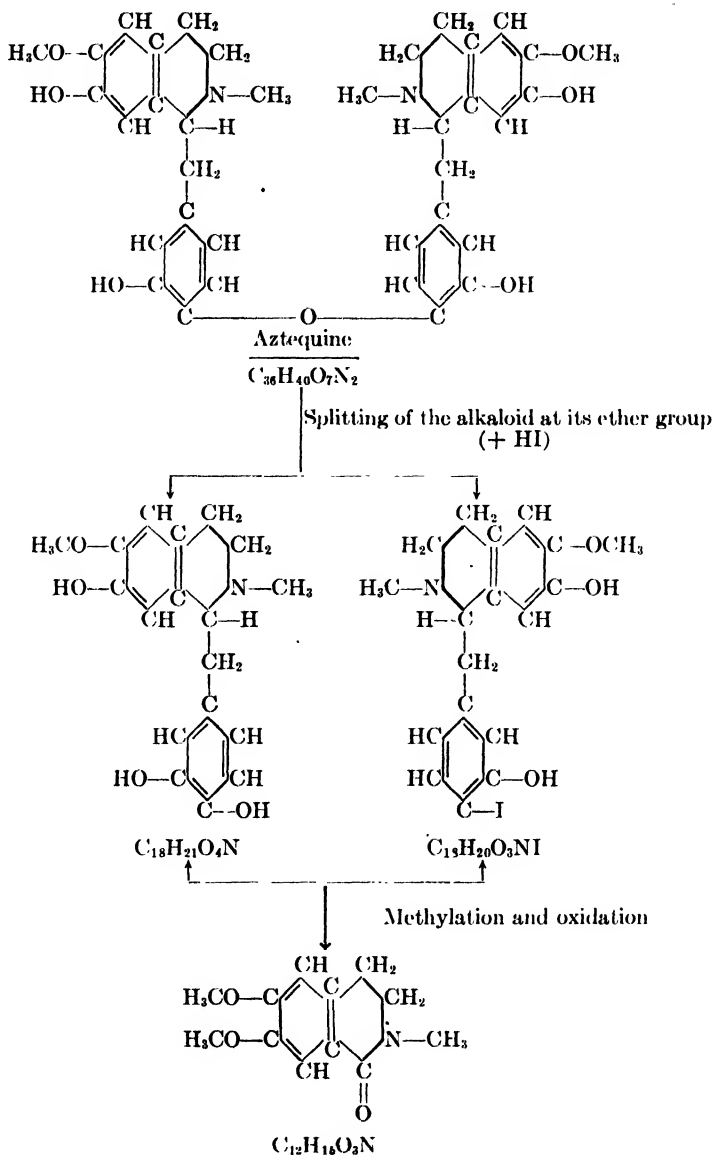
Yoloxochitl, the plant from which aztequine has been isolated, was used for centuries by the natives of Mexico for the treatment of certain syndromes similar to that of cardiac failure. The chemical structure of the alkaloid does not suggest that it may exert any digitalis-like action.

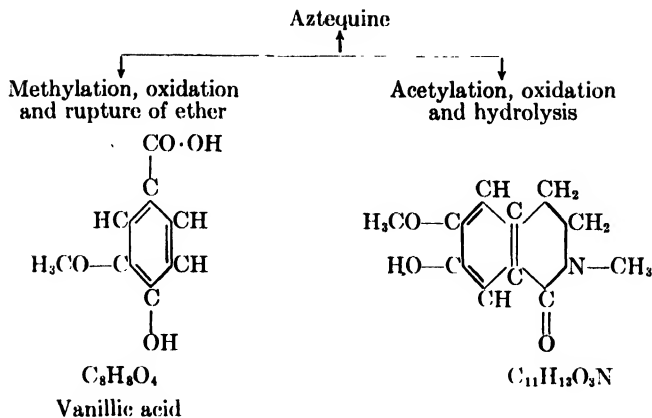
Nevertheless, a study of its effect on the isolated frog heart has been carried out by Dr. Rafael Méndez.

The hearts were isolated according to the Straub-Fühner technique. A concentration of  $1 \times 10^{-3} M$  caused an immediate and profound depression of the heart beat. this diastolic effect was reversed by washing with Clark's solution.

A concentration of  $1 \times 10^{-4} M$  caused no appreciable effect but the repeated replacement (two to four times) of the above concentration caused a gradually developing decrease in the amplitude of contraction accompanied by dropped beats. Finally the heart stopped in diastole. These effects were reversible.

We now present the structural formula of the alkaloid and the phase sequence by which we finally obtained it.





## ACKNOWLEDGMENT

We wish to express our sincere appreciation to Drs. Rafael Méndez, Frederick C. Uhle, and Antonio Madinaveitia for their helpful advice.

## SUMMARY

A technique is described by means of which a phenolic alkaloid of the isoquinoline group with an ether linkage was extracted from the leaves of the yolochochitl. Its empirical formula is  $C_{36}H_{40}O_7N_2$ . According to its chemical structure this alkaloid should be called the *para* ether bimolecule of *m*-hydroxyphenyl-2-methyl-7-hydroxy-6-methoxy-1,2,3,4-tetrahydroisoquinoline-1-(il)-methane. We propose the name "Aztequine" for this alkaloid.

The pharmacodynamic studies carried out with this alkaloid failed to show a digitalis-like action on the isolated heart of the frog.

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# The Preparation of Mushroom Tyrosinase

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Received August 13, 1947

## INTRODUCTION

Several requests have been received for more detailed information than contained in previous publications from these laboratories (1, 2, 3), concerning the procedures employed for obtaining highly active preparations of tyrosinase from the common mushroom, *Psalliota campestris*. As emphasized elsewhere (5), crude and purified preparations of the enzyme possess two different kinds of oxidase activity and the ratio of the two activities may be varied during the process of purification. Consequently, the activity properties of the purified enzyme depend very markedly on the procedures of salt fractionation, adsorption, *etc.*, used during the purification process.

The two activities that are characteristic of mushroom tyrosinase are: first, the ability to catalyze the aerobic oxidation of certain monohydric phenols such as tyrosine, phenol or *p*-cresol; and secondly, the ability to catalyze the aerobic oxidation of certain *o*-dihydroxyphenols such as catechol or homocatechol. The initial steps in the two oxidations appear to be quite different in character. The oxidation of a monohydric phenol involves the insertion of a hydroxyl group *ortho* to the one already present, while the oxidation of an *o*-dihydric phenol involves merely the removal of two hydrogen atoms to produce the corresponding *o*-quinone.

It has become the practice in these and other laboratories to use *p*-cresol and catechol as the experimental substrates for measuring the monohydric phenol and the *o*-dihydric phenol oxidase activities of

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tyrosinase. Consequently the two activities have been termed cresolase and catecholase activity, respectively. Purified preparations of the enzyme have been termed high catecholase or high cresolase preparations of tyrosinase depending on the ratio of the catecholase to cresolase activity in the purified preparation. Both types of preparations can be obtained from the same supply of the common mushroom, *Psalliota campestris*, and the procedures described below are typical of those used in these laboratories during the past few years.

### HIGH CRESOLASE PREPARATION (C 189)

#### *Extraction*

Thirty-six pounds of mushrooms were minced into 100 pounds of acetone, previously chilled with dry ice, and the pulp collected by filtration. After freezing the pulp for 4 hours with dry ice, the cake was broken up, suspended in about 25 l. of water and allowed to stand overnight in the refrigerator. The mixture was then filtered and the pulp pressed dry to recover as much activity as possible. This crude aqueous extract, amounting to 28 l., contained 560,000 cresolase and 280,000 catecholase units (see analytical section for methods of measuring activities).

#### *Separation of High Cresolase and High Catecholase Fractions*

The addition of sufficient ammonium sulfate to make the above solution 0.3 saturated at room temperature caused a precipitate to form which was removed by suction filtration.<sup>3</sup> This procedure was repeated, making the respective filtrates as they were obtained, 0.4 and 0.6 saturated with ammonium sulfate. The three resulting precipitates thus obtained were subsequently used for the preparation of enzyme high in catecholase activity. Aqueous solutions of these precipitates, about 400 ml. in each case, contained for the 0.3, 0.4 and 0.6 saturation, respectively, 8,000, 34,000 and 54,000 cresolase and 7,600, 31,200 and 76,000 catecholase units.

#### *Isolation and Further Purification of the High Cresolase Fraction*

The filtrate from the above 0.6 saturation with ammonium sulfate was then made 0.7 saturated with ammonium sulfate. The filtrate was discarded and the precipitate, collected by filtration as above, was dissolved in water to make 700 ml. of a solution containing 200,000 cresolase units and 40,000 catecholase units. Overnight dialysis of this solution against cold running tap water reduced the measured enzymatic activities to 185,000 cresolase and 35,000 catecholase units in a volume of 730 ml. (activity ratio, cresolase/catecholase = 5.6).

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<sup>3</sup> All suction filtrations throughout the course of preparation were carried out using a quarter-inch pad of celite (Johns Manville, No. 535) on the filter paper in the Buchner funnel. In most cases the precipitated protein was separated from the celite by dissolving in water and refiltering. After alumina adsorptions the precipitates were eluted from the pad and alumina using 0.2 M disodium phosphate solution.

The turbidity formed on adding 220 g. of barium acetate monohydrate to the above dialyzed solution was removed by filtration and discarded. Then 132 ml. of alumina reagent, prepared according to Willstätter (4), were added to the filtrate to remove color and extraneous protein matter and the resulting mixture was again filtered.<sup>4</sup> The filtrate contained 131,000 cresolase and 15,400 catecholase units in a volume of 1,440 ml. (activity ratio = 8.8).

The major part of the enzyme in the above filtrate was removed by adsorption on alumina in two stages. First, 100 ml. of molar barium acetate and 72 ml. of the alumina reagent were added and the mixture was filtered. The precipitate was saved (see below) and the filtrate contained 80,000 cresolase and 13,400 catecholase units

TABLE I

*Analytical Data on Two Tyrosinase Preparations from the Common Mushroom Psalliota campestris*<sup>a</sup>

Preparation number	Copper	Activity units/ml.		Units/γ Cu		Units/mg. dry weight		Activity ratio	Type
		Cat.	Cres.	Cat.	Cres.	Cat.	Cres.	Cat./Cres.	
C21 I-228 F2	<i>Per cent</i>								
	I 0.206	21,500	450	2130	48	4400	95	48	High Catecholase
	II 0.204	49,000	1140	1980	47	4050	95	43	
C 189	0.028	1,880	1,180	856	536	237	149	1.6	High Cresolase

<sup>a</sup> All catecholase and cresolase activities, recorded in Table I, were determined using the methods described in the preceding paragraph. (See section on Analytical Details.) For this reason the activity ratios do not correspond to those in the text.

in a volume of 1,580 ml. (activity ratio = 6.0). Then 158 ml. more of the alumina reagent was added to the filtrate and the mixture again filtered. The precipitate was saved (see below) and the filtrate contained 40,000 cresolase and 6,800 catecholase units in a volume of 1,700 ml. (activity ratio = 5.9).<sup>5</sup>

The two fractions adsorbed on alumina (the solids collected in the two preceding steps) were combined and added with stirring to 0.2 *M* disodium phosphate to elute the enzyme. Filtration produced 380 ml. of solution containing 74,000 cresolase and 13,300 catecholase units which were then dialyzed against cold distilled water. The

<sup>4</sup> Although the precipitate contained considerable adsorbed enzyme activity, it was not used further because of its relatively higher catecholase: cresolase activity ratio than that of the enzyme in the filtrate.

<sup>5</sup> The filtrate at this stage usually was not further purified. Although it contained about 30% of the cresolase activity present before the two alumina adsorptions, the enzyme was considerably less pure than the adsorbed fractions.



dialyzate contained 75,000 cresolase and 13,000 catecholase units in a volume of 410 ml. (activity ratio = 5.8). The addition of 41 ml. of the alumina reagent and 41 ml. of 0.1 saturated lead subacetate solution, followed by filtration and elution of the precipitate with 0.2 *M* disodium phosphate yielded 77 ml. of enzyme solution containing 48,000 cresolase and 8,300 catecholase units (activity ratio = 5.8). The enzyme was further concentrated by making the above solution 0.6 saturated in ammonium sulfate, filtering, and dissolving the precipitate in a little water and dialyzing. In this way 15 ml. of a high cresolase preparation of tyrosinase was obtained which contained 7.94 mg. (dry weight) of undialyzable solid/ml., and a total of 48,000 cresolase and 8,500 catecholase units (activity ratio, cresolase/catecholase = 5.6).

The cresolase activity of preparation C 189, measured by the method of Mallette and Dawson (5), was 1,180 units per ml., and the catecholase activity measured by the chrometric method (6) was 1,880 units/ml. (see analytical section). The preparation had a copper content of 0.028% and proved to be about 70–80% electrophoretically homogeneous at pH 7.71 (7). The analytical data on preparation C 189 are summarized in Table I.

### *High Catecholase Preparation (C 211–228 F2)*

As stated above in the section on *Separation of High Cresolase and High Catecholase Fractions*, the precipitates obtained by making the extract of the frozen minced mushrooms 0.3, 0.4, and 0.6 saturated with ammonium sulfate were reserved for obtaining preparations high in catecholase activity.<sup>6</sup> Eighteen such precipitates which had accumulated in the laboratory over a short period of time were dissolved in water and combined, yielding 5 liters of a solution containing 655,000 cresolase and 1,726,000 catecholase units (activity ratio, catecholase/cresolase = 2.6). The precipitate obtained from this solution, after the addition of ammonium sulfate to 0.5 saturation, was dissolved in 1,600 ml. of 0.2 *M* disodium phosphate and dialyzed overnight against running tap water. The dialyzed solution contained 525,000 cresolase and 1,665,000 catecholase units in a volume of 1,800 ml. (activity ratio = 3.2).

### *Further Fractionation of the Catecholase and Cresolase Activities*

To remove color and further fractionate the catecholase and cresolase activities the above dialyzed solution was treated with 36 ml. of the alumina reagent and then filtered.<sup>7</sup> The filtrate contained 154,000 cresolase and 800,000 catecholase units in 2,200 ml. (activity ratio = 5.2). The enzyme in the filtrate was then adsorbed by the addition of 220 ml. more of alumina reagent and, after filtration, the enzyme was eluted from the alumina by stirring in 0.2 *M* disodium phosphate. The 270 ml. of solution obtained was dialyzed overnight against running tap water and found to

<sup>6</sup> In some cases, depending on the particular batch of mushrooms used, it was found better to use the precipitate from the 0.6 saturation for the preparation of high cresolase rather than using it for the high catecholase preparation.

<sup>7</sup> Although the precipitate contained considerable adsorbed enzyme activity, it was not used further because of its relatively lower catecholase to cresolase activity ratio than that of the enzyme in the filtrate.

contain 84,500 cresolase and 621,000 catecholase units (activity ratio = 7.3). The careful addition of 0.1 saturated lead subacetate solution caused a considerable precipitation of extraneous protein matter which was removed by filtration. The enzyme in the filtrate (450 ml.) was then adsorbed on alumina (45 ml.), filtered, and eluted by stirring with 0.2 *M* disodium phosphate solution. The eluate contained 100,000 cresolase and 660,000 catecholase units (activity ratio = 6.6) in a volume of 110 ml. This solution was then made 10% in acetone, and 0.1 saturated lead subacetate was added until no further precipitation occurred. After filtration, the precipitate was mixed with about 35 ml. of 0.2 *M* secondary phosphate, filtered again, and dialyzed against several changes of distilled water. The dialyzate had a volume of 40 ml. and contained 640,000 catecholase units.

### *Fractionation with Lead Subacetate Solution*

The above solution was made 10% in acetone and precipitated in four fractions by the portionwise addition of 0.1 saturated lead subacetate solution. The enzyme in each of the four precipitates was eluted by a small volume of 0.2 *M* disodium phosphate, dialyzed, and assayed for enzymatic activities and dry weight. The solution from the second precipitate (18 ml.) contained the most enzyme and in the best degree of purity. It contained 50,000 cresolase units, 347,000 catecholase units (activity ratio = 6.9) and 12.8 mg. (dry weight) of undialyzable solids per ml.

A 6 ml. portion of the above enzyme solution was filtered through a porous bottom porcelain crucible, diluted to 15 ml., and dialyzed against 0.05 *M* phosphate buffer of pH 7.58. This preparation (C 211-228 F2-I) had a copper content of 0.206% and proved to be electrophoretically homogeneous at pH 7.58 (7).

The remaining 11 ml. of the above enzyme solution were also filtered through a porous bottom porcelain crucible and dialyzed against 0.05 *M* phosphate buffer of pH 7.67 without first diluting the sample. This preparation (C 211-228 F2-II) had a copper content of 0.204% and proved likewise to be electrophoretically homogeneous at pH 7.67 (7). The analytical data on preparation C 211-228 F2 are summarized in Table I.

## ANALYTICAL DETAILS

### *Activity Measurements*

All activities recorded during the course of the purification processes described above were made using the catecholase (catecholhydroquinone) and cresolase activity methods of Adams and Nelson (8), except that in the cresolase method the reaction flask also contained 0.1 mg. of catechol to remove the induction period. Although these methods have been criticized (6, 9) and are not suitable for comparing the properties of purified preparations, they have the great advantage of being relatively simple and quite rapid. For these reasons they were used throughout the preparative procedures where very often speed of operation is more essential than a precise measurement of the activity.

The activities of the final purified preparations, as listed in Table I, were measured using the manometric cresolase method as modified by Mallette and Dawson (5) and the chronometric catecholase method (6).

#### *Dry Weight Determinations*

Aliquots of each preparation were obtained salt-free by dialysis for 3–5 days in cellophane bags using 10–15 changes of copper-free water. The dialysis bags were then opened and the contents transferred quantitatively with thorough washing into volumetric flasks. After making to volume and mixing, an aliquot of each of these dialyzed solutions was evaporated to dryness, weighed, and the dry weight of the original sample calculated. When a polarographic determination of the copper content was made, the residues from these dry weight determination were employed as the samples. The actual evaporation to dryness in the determination of dry weights was carried out at 100°C. in thin bulbs blown of pyrex glass, each having a capacity of about 5 ml. There was no need to seal the bulbs after heating, since the dried protein was not hygroscopic. The bulbs and contents were heated with a slow stream of air (dried with sulfuric acid) blown through a capillary into the bulb. Twenty minutes after the last visible moisture had disappeared, the heating was discontinued and the bulbs were cooled over calcium chloride for 30 minutes. They were then weighed to the nearest 0.01 mg. using a semimicro balance. Duplicate determinations usually agreed to within less than  $\pm 0.02$  mg., and reheating for a short period had little effect on the weight. However, reheating for several hours caused a considerable darkening and some loss in weight, probably due to decomposition of the protein.

#### *Copper Determinations*

The copper data listed in Table I were obtained using the polarographic method of Ames and Dawson (10). Preparation C 189 was also assayed by the manometric method of Warburg and Krebs (11) based on the copper catalysis of the oxidation of cysteine. The latter method gave a copper content of 0.029% for preparation C 189.

#### ACKNOWLEDGMENT

The authors wish to express their thanks to the Rockefeller Foundation for a grant supporting part of this work, and to Dr. D. H. Moore, College of Physicians and Surgeons, Columbia University, for the electrophoretic analyses.

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# Oxygen Absorption Method for the Estimation of Coenzyme I<sup>1</sup>

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Received August 18, 1947

## INTRODUCTION

The use of enzyme systems in the estimation of coenzyme I has been critically reviewed by Sumner and Krishnan (1) in a recent publication. They also reported the use of two systems for coenzyme I estimation; in one, lactic apodehydrogenase, lactate, cyanide, diaphorase, and methylene blue are employed, and in the other washed brewer's bottom yeast, hexose diphosphate, phosphate buffer, and methylene blue. In the latter system hexose diphosphate is split by aldolase present in the washed yeast into triose phosphates; 3-phosphoglyceric aldehyde, one of the products formed, is phosphorylated to 1,3-diphosphoglyceric aldehyde; triosephosphate-apodehydrogenase, another enzyme present in the washed yeast, now oxidizes the above compound to 1,3-diphosphoglyceric acid, if coenzyme I is added. When methylene blue is present, the course of the reaction can be followed either visually by noting the bleaching under anaerobic conditions, or by observing the oxygen absorption in the Warburg manometer, the reaction taking place in contact with atmospheric air. The details for the first method have been described in the publication referred to above; the following is an account of the manometric method of estimation. The experiences in this laboratory in the drying of brewer's yeast, washing of the dried yeast for the removal of coenzyme I, and the storage of such washed samples, are also being recorded.

<sup>1</sup> Published as short note in *Science* 105, 295 (1947).

## EXPERIMENTAL

*The Test System*

The test system, as finally worked out, has the following composition:

*Side arm.* 0.1 cc. methylene blue, 1 in 100,

0.1–0.4 cc. coenzyme I solution, containing 4–16  $\gamma$ ,

Water added to 0.5 cc.

*Main flask.* 0.3 cc. 0.5 M phosphate buffer, pH 8.0,

0.5 cc. Harden and Young ester (corresponding to 8–10 mg. of the barium salt),

1.0 cc. of washed brewer's bottom yeast, corresponding to 200 mg. of the original dried yeast,

0.1 cc. Mg, Mn mixture, containing 0.5 mg. of each.

Water added to make 2.3 cc.

*Central cup.* 0.2 cc. 20% potassium hydroxide.

Total volume 3 cc.

Temperature of bath 38°C.

Atmosphere air.

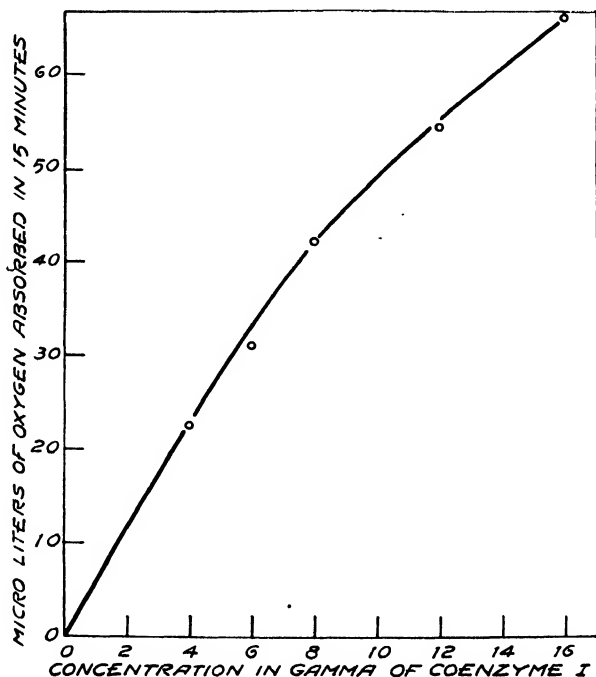


FIG. 1. Relationship between the concentration of coenzyme and the oxygen absorption.

The flasks are allowed to equilibrate with shaking for 15 minutes, during which time the aldolase splits the ester. The coenzyme and methylene blue are now tipped from the side arm, the flasks returned to the bath, and the shaking continued. After 5 minutes, when the flasks have regained equilibrium, the zero reading is taken; the shaking is allowed to continue for exactly 15 minutes more and the readings again noted. When the oxygen absorption during the 15 minutes' period, after correcting for blank absorption, is plotted against the concentration in  $\gamma$  of coenzyme, a graph is obtained (Fig. 1), which is practically a straight line for concentrations of coenzyme up to 8  $\gamma$  and thereafter curves off. This graph can be used for evaluating the potency of unknown solutions.

### *Mode of Drying of Yeast*

Brewer's bottom yeast used in our experiments was the *Lager* variety supplied by Haberle Congress Brewing Co., Syracuse, New York. *Ale* yeast was found to be unsatisfactory for the purpose. Our experiments lead us to the conclusion that the potency of dried preparations is dependent, to an extent, on the mode of drying. The maximum potency was obtained when the washed pressed yeast was dried in shallow layers for about 24 hours at a temperature of about 20°C. We have designated this as our standard preparation. When the drying is carried out more rapidly and at a temperature of about 30°C., by placing the sieved yeast under a warm-air blower, the potency of the resulting specimen is definitely lower. Whereas 200 mg. of the standard specimen of yeast gave maximum oxygen absorption with 8  $\gamma$  of coenzyme I, the same amount of the preparation dried at the higher temperature was found to be insufficient to give the maximum absorption, the respective figures being 40.5 and 36.3  $\mu$ l. during a period of 15 minutes. Another specimen was dried at a temperature of 7°C.; the oxygen absorption in this case was only 30.7  $\mu$ l., indicating that there has been appreciable loss in potency. This is not difficult to understand, because drying at low temperatures takes a few days for completion and autolysis is likely to set in, so that, on subsequent washing, some of the enzymes might be washed out. When a fourth specimen was dried *in vacuo*, at the ordinary temperature, a sample was obtained, which was characterized by very high blanks, indicating that the coenzyme could not be satisfactorily washed out.

"Maceration juice" and "Zymin" prepared from brewer's yeast, according to standard methods (2), were found to be unsatisfactory as the source of enzymes. Experiments were also carried out with baker's yeast; one sample was dried by the method of Govier (3) and then



washed with water; such a specimen had only a feeble activity. Nor was autolyzed baker's yeast suited for the purpose.

The method that was finally adopted is as follows:

Fresh brewer's bottom yeast, transported in ice, is centrifuged in the cold and the residue washed with large volumes of ice cold distilled water, any hops and other extraneous matter being removed by taking advantage of the fact that they settle out more rapidly. The washing is carried out three times in the cold room. On a small scale the centrifuge can be used conveniently, but when large amounts are being handled it is best to allow the suspension to settle overnight and siphon off the supernatant. The final residue is filtered with suction on the Buchner funnel and pressed hard. The solid is forced through a sieve with 45 holes per square inch, the material being allowed to fall in a very thin layer on sheets of brown paper and allowed to dry in a big room with good air circulation. The drying is practically complete overnight, but is usually allowed to proceed for a few hours more (not more than 24 hours total). A pale brown gritty solid is obtained, which has a moisture content of about 6%. The material is stored in stoppered bottles in the desiccator, in the cold room.

### *Stability of Dried Yeast*

Stored in the cold, the activity of the preparation was unchanged for 6-8 weeks. Different preparations of etiozymase prepared from the same batch of dried yeast yielded practically the same oxygen absorption with a given quantity of coenzyme I. Thus, three samples of washed yeast prepared during intervals of a few weeks gave 42.1, 42.8, and 40.5  $\mu$ l. of oxygen absorption in 15 minutes with 8  $\gamma$  of coenzyme. Also, different batches of brewer's yeast procured at different periods from the same brewery gave practically the same oxygen absorption. Storage for more than 2 months was attended with detectable loss in potency, and, at the end of 7 months, 200 mg. of yeast was no longer sufficient to give the maximum absorption. A sample of dried yeast which had been stored in the cold for 2 years was found to be quite useless as the source of etiozymase, although the unwashed yeast still retained the capacity of rapidly fermenting added glucose.

### *Preparation of Coenzyme-Free Yeast*

When dried brewer's yeast is washed with large volumes of distilled water, a product is obtained, "Apozymase," which is found to be highly active when tested in the system outlined above, but the results are vitiated by the fact that the blanks are very high. Dialysis of such preparations against distilled water in the cold was without any ap-

preciable effect on the blank absorption. Drying of the material and rewashing with water was attended with considerable loss in potency. Another disadvantage of working with apozymase is the rapid loss in potency even on storage in the cold. Washing of dried yeast with reagents like dilute alcohol, dilute acetone, aqueous ether or with very dilute solutions of cysteine, *etc.*, yielded preparations which had either too high a blank or too low a potency. We have finally come to the conclusion that the most satisfactory results are obtained when buffers are used for the washing. Acid phosphate buffers yielded products which had low blank absorption, but the activity was not as high as could be desired. "Etiozymase," obtained by washing the dried yeast with alkaline phosphate buffer, was found to be quite satisfactory, the blanks being low and the potency at an optimum.

The washing is carried out as follows. Ten g. of the dried yeast is stirred mechanically for 15 minutes with distilled water at ordinary temperature and centrifuged for 30 minutes in the cold room. The residue is suspended in 500 cc. of 0.2 *M* phosphate buffer, pH 7.8, and stirred mechanically for 30 minutes; the centrifuged residue is washed once again with the buffer and then twice with water. The final residue is stirred up with water and the volume made up to 50 cc., so that each cc. of the suspension would correspond to 200 mg. of the original dried yeast. The pH of the suspension was 6.3–6.4; 1 cc. aliquots on drying for 2 hours at 80°C. gave a residue of 125 mg.

### *Stability of Washed Yeast*

As already mentioned, apozymase obtained by washing dried yeast with water shows rapid deterioration in potency on storage even in the cold. When, however, phosphate buffer is used for the washing, the etiozymase so obtained is much more stable on storage. We have preferred to store our samples frozen, usually after distributing into a number of containers. Just before use, the material is allowed to thaw and shaken up to get a uniform suspension. The washed yeast is serviceable for about a week.

### *Optimum Amount of Yeast for Each Estimation*

We have found that 1 cc. of the freshly prepared suspension, corresponding to 200 mg. of the original dried yeast, is sufficient to give maximum oxygen absorption with the various concentrations of coenzyme that we have employed; 0.5 cc. was insufficient and 1.5 cc. did not give any increased absorption. Of course, in the case of compara-

tively inactive preparations 1.5 cc. or more of the suspension would have to be employed in each reaction flask.

#### *Influence of Temperature on the Steadiness of Oxygen Absorption*

When the reaction is carried out at a bath temperature of 25°C., and the manometer readings taken every 10 minutes, it is found that the oxygen absorption for a given concentration of coenzyme is steady for a period of about 40 minutes, after which time the rate of absorption gradually falls. At a bath temperature of 38°C. the rate of absorption is speeded up, but it is steady only for a period of about 20 minutes, after which there is a decline, due partly to the instability of the enzymes at the higher temperature and possibly increased destruction of coenzyme I. In all our experiments, therefore, where the reaction was carried out at 38°C., we have measured the oxygen absorption for the first 15 minutes only.

#### *Influence of pH on the Reaction*

We have tested the effect of three different phosphate buffers; with a given concentration of coenzyme the oxygen absorption at pH 9.3 was 41.6  $\mu$ l., at pH 8.0 it was 42.8, and at pH 7.4 it was only 32.1  $\mu$ l. It is evident that the reaction is definitely retarded at pH 7.4; the absorption is practically the same at pH 8.0 and at pH 9.3, but we have preferred to use the lower of the two in view of the known instability of oxidized coenzyme I toward alkali.

#### *Period of Action of Aldolase*

We usually allow the washed yeast to incubate with hexose diphosphate for 15 minutes at 38°C. Longer periods of incubation at the same bath temperature result in lowered oxygen absorption, due probably to the inactivation of triosephosphateapodehydrogenase.

#### *Decomposition of Coenzyme I by Yeast*

It is a known fact (4) that coenzyme I is very rapidly inactivated by incubation with apozymase alone, but that it remains intact if phosphate buffer, glucose, and hexose phosphate are also present. We find that when coenzyme I is incubated in the main flask with the washed

yeast, phosphate buffer, and hexose diphosphate at 38°C. for 45 minutes, and the methylene blue then added from side arm, the oxygen absorption is reduced to about 50%, which is due partly to destruction of coenzyme and partly to inactivation of enzymes.

#### *Influence of Added Magnesium and Manganese*

In our test system we have been adding 0.5 mg. each of magnesium and manganese. Nilsson (5) has pointed out that in the fermentation test with apozymase, the absence of magnesium, or its presence in insufficient amounts, leads to extremely erroneous results. When these inorganic ions are withheld from the system outlined above, the oxygen absorption is reduced by only 10%, a result which is in keeping with the fact that neither aldolase nor triosephosphateapodehydrogenase requires Mg or Mn for activation.

#### *Effect of Enzyme Poisons*

In separate experiments the oxygen absorption was measured in the presence of 0.3 cc. of 5% arsenate, 10% fluoride, 10% iodoacetate, and 5% azide. Arsenate and fluoride were without marked inhibiting effect on the absorption of oxygen, but with azide there was an inhibition of about 30–40%, and with iodoacetate the reaction was practically completely inhibited. It is well known that iodoacetate acts as a powerful inhibitor for diphosphoglyceric aldehyde dehydrogenase.

#### ACKNOWLEDGMENT

The author's thanks are due to Professor James B. Sumner for his interest in the investigation, to the Haberle Congress Brewing Co., Syracuse, New York, for generous supplies of yeast, and to the Rockefeller Foundation for financial aid.

#### SUMMARY

Coenzyme I can be estimated conveniently and accurately by oxygen absorption in the Warburg manometer using the following system:

washed brewer's bottom yeast,  
hexose diphosphate,  
phosphate buffer,  
methylene blue.

The processing of brewer's yeast for the preparation of coenzyme I-free material has been studied.

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# The Isolation of Inulin and an Unidentified C<sub>20</sub> Compound from the Roots of *Solidago canadensis* L.

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Received August 26, 1947

## INTRODUCTION

In the course of the investigation of the chemical composition of several common weeds, roots of goldenrod (*Solidago canadensis* L.) were examined. The following report deals with the isolation of inulin and of an unidentified C<sub>20</sub> compound from such material.

## EXPERIMENTAL

### *Inulin*

Although inulin is recognized as of quite general occurrence in members of the family *Compositae*, no reference to its actual isolation from goldenrod could be found.

Roots of *Solidago canadensis* L. gathered in October, 1946, were air-dried and ground to about 20 mesh in a Wiley mill. About 970 g. of root meal were thus obtained. This was exhaustively extracted in a continuous type extraction apparatus with 3 liters of petroleum ether followed by approximately the same amount of acetone and finally by 95% ethyl alcohol. These extracts were set aside for further investigation.

The extracted meal was placed in a cloth bag and immersed in 3 liters of boiling distilled water for an hour. The extraction was repeated with another 3 liters of distilled water and the extracts combined. This water solution was clarified by treatment with a small volume of neutral lead acetate solution. De-leading was effected with hydrogen sulfide. The filtered liquid was concentrated to a volume of about 200 cc., and 500 cc. of 95% ethyl alcohol was added. A white granular

precipitate consisting of sphero-crystals resulted which gave the usual reactions for inulin. Twenty five grams of inulin were thus obtained, representing a concentration of about 2.6% of the dried roots.

#### *An Unidentified C<sub>20</sub> Compound*

The above described petroleum ether extract, which was deep yellow in color, was concentrated to a small volume. Crystals, which slowly formed, were filtered off and recrystallized 3 times from petroleum ether. There were thus obtained 1.64 g. of colorless, cubical crystals which melted at 89–90°C. By the Rast method a molecular weight of 300 was found. Analysis for carbon and hydrogen was as follows:

$$\begin{array}{l} \text{C}—75.84, 75.96\% \\ \text{H}—8.82, 8.90\% \end{array}$$

These data correspond to a formula of C<sub>20</sub>H<sub>28</sub>O<sub>3</sub>. No active hydrogen was present as indicated by failure to react with metallic sodium in anhydrous benzene. Refluxing for an hour with either acetic anhydride or 95% ethyl alcohol resulted in the formation of colorless needles melting at 131–132°C. However, since the molecular weight as well as the carbon and hydrogen analysis was the same as for the original compound, it is believed that the treatments merely produce a molecular rearrangement. No aldehyde or ketone groups could be detected. On warming a few crystals of this compound before or after the above treatments with a drop of concentrated sulfuric acid a deep red color resulted, a reaction characteristic of many terpene compounds. No compound having the properties described above could be found in the literature.

#### SUMMARY

The isolation of inulin and of an unidentified compound having the formula C<sub>20</sub>H<sub>28</sub>O<sub>3</sub> from the roots of *Solidago canadensis* L. is described.

# **The Effect of Extirpation of Various Endocrine Glands on the Production of Fatty Liver**

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Received September 5, 1947

## **INTRODUCTION**

Since absence of lipotropic substances in the diet is the leading etiologic factor in the development of dietary cirrhosis of the liver (1), and, in consequence, excess of liver fat characteristically precedes and accompanies this type of cirrhosis, it is of importance to examine all mechanisms which influence fat deposition in the organ. A lipotropic effect of estrone has been reported in a previous communication (2). The present study includes further experiments in which rats, subjected to operative extirpation of various endocrine glands, were placed on a diet low in lipotropic factors, and capable of inducing a fatty liver. The degree of fatty change was compared with that produced in intact rats by the same diet.

## **METHODS**

The rats were of a local hooded strain and ranged in weight from 180 to 250 g. Following operation they were maintained on Purina Dog Chow for 2-3 weeks until preoperative weight was regained. They were then given a low protein, high fat diet of the following composition: Casein 8%, sucrose 50%, hydrogenated vegetable oil 38%, salt mixture [modified McCollum (3)] 4%. Each animal also received daily 20  $\gamma$  thiamine chloride, 100  $\gamma$  calcium pantothenate, 20  $\gamma$  pyridoxine, and 25  $\gamma$  riboflavin in 1 ml. of water. Three drops of percomorph oil furnishing 3,750 units of vitamin A and 540 units of vitamin D, and 3 mg. of  $\alpha$ -tocopherol were given weekly. Adrenalectomized rats received 0.9% sodium chloride as drinking water during the entire post-operative period.



After 21 days on the diet the rats were killed and the livers analyzed chemically for lipid content. The group of hypophysectomized rats was initially intended for a long-term cirrhosis experiment and hence the animals were not killed after 21 days but allowed to survive as long as possible. The period of survival varied from 64 to 108 days. All rats were checked at autopsy for completeness of removal of glandular tissue and any suspicious remaining material was examined microscopically.

Liver fat was determined as follows: Approximately 1 g. of liver was ground in a glass mortar and washed into a 125 ml. Erlenmeyer flask with successive portions of a 3:1 ethanol-ether mixture (50 ml. total). After standing overnight in the icebox the mixture was brought to boiling in a hot water bath, filtered through fat-free paper, and washed with three 25 ml. portions of warm alcohol-ether. After evaporation to dryness *in vacuo*, the residue was extracted with a total of 60 ml. of petroleum ether and the latter allowed to settle overnight in a 100 ml. stoppered graduate at icebox temperature. A 15 ml. aliquot was finally evaporated to dryness in a weighed beaker, first over a steam bath, and finally for 0.5 hr. in an oven at 60°C.

TABLE I

*The Effect of Glandular Extirpation on Liver Fat*

		Number of animals	Liver fat	Per cent wt. change	Av. daily food intake
Controls	♂	11	17.1±1.4	-5.1 ±1.0	9.6±0.45
	♀	12	13.2±1.5	+0.17±1.8	8.6±0.30
	Both sexes	23	15.2±1.1	-3.3 ±1.2	9.1±0.28
Thyrex	♂	10	9.2±0.9	-5.1 ±2.1	6.4±0.43
	♀	10	8.6±0.34	-4.1 ±2.3	6.2±0.37
	Both sexes	20	8.9±1.2	-4.7 ±1.5	6.3±0.28
Adrex	♂	10	8.7±1.6	-18.5±1.1	6.1±0.33
	♀	10	12.4±1.9	-11.6±1.9	6.1±0.34
	Both sexes	20	10.5±1.3	-15.1±1.3	6.1±0.23
Hypox	♂	2	6.6±1.0	-38.2±1.2	2.9±0.32
	♀	5	10.2±1.8	-32.4±2.2	3.0±0.27
	Both sexes	7	9.2±1.4	-32.5±2.0	3.0±0.18
Gonadex	♂	10	13.6±1.5	-5.1 ±1.4	8.2±5.8
	♀	10	21.2±3.4	-3.3 ±1.5	7.8±0.30
	Both sexes	20	17.4±2.0	-4.2 ±1.0	8.0±0.52
Normal rats on chow diet	♂	10	8.1±0.31		
	♀	10	7.0±0.32		
	Both sexes	20	7.5±0.24		

## RESULTS

The most striking alteration in liver fat was encountered in the group of thyroidectomized rats (Tables I and II). The values in this group were distinctly lower than in the series of control rats on the experimental diet and, in fact, approached the range of values obtained in animals receiving a normal diet of Purina Chow. Males and females were equally affected by thyroidectomy. The average food consump-

TABLE II

*Summary of Effects of Removal of Endocrine Glands on Liver Fat*

	Total series	Male	Female
Thyroid	Decrease	Decrease	Decrease
Adrenal	Decrease	Decrease	No significant change
Pituitary	Decrease		
Gonads		No significant change	Increase

tion of the animals was lower than the controls but there was no significant difference in weight loss. The combination of low food consumption and minimal weight loss is compatible with the low metabolic rate of the thyroidectomized animal.

Adrenalectomy resulted in a significant reduction of liver fat in the male but not in the female rats. The adrenalectomized rats ate significantly less food than normal animals and their weight loss was fairly great.

The liver fat content in the small series of hypophysectomized animals was significantly low. Food intake of this group was very low and weight loss was extreme. Such a degree of weight loss may in itself prevent fat from accumulating in the liver (4), and the results must, therefore, be interpreted with caution.

No significant effect was produced by removal of the testes. Ovariectomy on the other hand was followed by a substantial increase of fat above control levels. A similar tendency has been noted in previous

experiments (2), although the present results represent the first observations which bear up under statistical analysis (Table III).

TABLE III  
*Significance of Difference from Controls*

	Total series			Males			Females		
	Diff. and error	<i>t</i>	<i>p</i>	Diff. and error	<i>t</i>	<i>p</i>	Diff. and error	<i>t</i>	<i>p</i>
Hypox	-6.0±1.7	3.3	<0.01						
Adrex	-4.7±1.7	2.7	<0.01	-8.4±2.1	3.9	<0.01	-0.8±2.4		
Thyrex	-6.3±1.6	3.8	<0.01	-7.9±1.6	5.9	<0.01	-4.6±1.5	3.0	<0.01
Gonadex				-3.5±2.1	1.6	>0.05	+8.0±3.7	2.1	<0.05

## DISCUSSION

The experimental diet which was employed causes a fatty metamorphosis of the liver and, after prolonged administration, cell damage with cirrhosis. If fat deposition is diminished by hormone deprivation during such a dietary regime one might suspect a corresponding amelioration of liver damage. It is possible, however, that the mechanism is not actually a protective one. The hormone may be involved in the physiological processes of fat synthesis or transport and have no effect on the development of cirrhosis.

Accumulation of fat is closely related to cell damage, but experimental data (5, 6) have demonstrated that the two processes may vary independently. It is by no means established that cirrhosis must follow fatty infiltration of the liver. Evidence has been adduced, nevertheless, that in clinical hyperthyroidism liver function is often impaired and actual cirrhosis may occur (8). Although the liver damage of hyperthyroidism has recently been attributed to hemodynamic alterations (9), it is possible that a metabolic aberration could be of prime importance in its pathogenesis. Not only is liver glycogen low, but protein catabolism is increased, and all protective factors might be depleted by the augmented cellular metabolism. In contrast, the protection against fatty liver afforded by thyroidectomy might be ascribed to a sparing effect of hypometabolism on lipotropic substances such as methionine.

György and Goldblatt have shown that thiouracil exerts a well marked protective effect against dietary cirrhosis in the rat (10). It may be reasoned either that such a drug acts directly on the liver or that the action is indirect and results from the induced hypothyroidism. Inasmuch as thyroidectomy protects against fatty meta-

morphosis it seems likely that the beneficial effect of thiouracil in preventing cirrhosis is likewise dependent on the accompanying depression of thyroid function, and one would suspect that thyroidectomy in turn would protect against cirrhosis.

In the experiments here reported thyroidectomy appeared to counteract the effect of an anti-lipotropic diet. Similar reduction of the total fatty acids of the liver below normal level (with slight elevation of the cholesterol content) has been found by Artom (11) in 1923 following thyroidectomy in animals of various species (dogs, rabbits, guinea pigs) kept on a normal laboratory ration.

According to May, Moseley and Forbes (12) administration of thiourea in doses up to 1% in the diet does not affect the distribution of neutral fat and cholesterol in the liver of rats. However, it should be borne in mind that in these studies the experimental diet, even when low in protein, contained enough choline to counteract its possible anti-lipotropic potency. In earlier experiments Forbes (13) found thyroxine without effect on liver fat in rats fed a "high carbohydrate, low protein, fat free" diet, but the hormone appeared to potentiate the lipotropic effect of choline.

Handler recently made a preliminary report (14) on experiments in which he studied the effect of supplements of thyroid substance and of thiouracil on liver lipids in rats fed a ration similar to ours. He found that feeding of thyroid substance decreased, whereas thiouracil, "by depressing thyroid activity," increased lipid deposition in the liver. These results seem to be at variance with our own conclusions, and to some extent also with those of the investigations mentioned above. Several explanations may be given for these discrepancies, but until they have experimental confirmation they must be regarded as suggestions only. In Handler's rats thiouracil may not have been given long enough to induce a hypothyroidism equivalent to that produced by total thyroidectomy in our own animals, or the dose may have been so high as to be toxic. Conversely, administration of thyroid substance may have caused in his animals marked tissue destruction and weight loss, both of which are known to militate against the development of fatty liver. On the other hand, it is possible that, where thiouracil is given as a supplement to a synthetic diet low in methionine and choline, its protective effect on cirrhosis does not exactly parallel its effect on liver fat. In the experiments of György and Goldblatt (10) the histologically demonstrable fat in the liver did not appear to be reduced by thiouracil in spite of significant amelioration of the cirrhotic process.

The anterior pituitary and adrenal cortex are both recognized as glands which take part in the control of liver fat. Pituitary extract is known to produce a fatty liver (15). It has been shown by deuterium studies that the fat found in the liver cells after the administration of pituitary extract is from the peripheral deposits, but that the fat which appears during the feeding of a diet low in lipotropic factors does not arise from this source (16, 17). Such findings do not favor the existence of closely parallel metabolic pathways involving dietary and pituitary hormonal mechanisms.

Although it has been reported that hepatic cirrhosis may follow hypophysectomy in dogs, it was subsequently demonstrated that these lesions are correlated with hypothalamic damage and not with the absence of the pituitary (18). Hypophysectomy in the rat (performed by the parapharyngeal approach) has been shown to reduce the liver fat in animals on a high fat or high carbohydrate diet (19).

That the adrenal cortex is involved in the control of liver fat is evidenced by previous work which indicates that fatty liver due to a variety of causes may be prevented by adrenalectomy (20, 21). MacKay (21) found that fat left the liver more rapidly in rats which had previously been given a low protein diet if the animals were adrenalectomized. More recently, Hartman (22) has obtained evidence that there may be a specific hormone ("fat factor") of the adrenal cortex which differs from the other adrenal steroids and which promotes fatty metamorphosis of the liver.

The marked weight loss following adrenalectomy and hypophysectomy in rats does not allow a definite conclusion as to the true protective effect of the relevant surgical procedures on the production of fat infiltration of the liver. This possible source of error notwithstanding, in our own experiments the statistically significant decrease in liver fat after adrenalectomy in the male rats, in contrast to the less distinct reduction of liver fat in the female animals, deserves special interest.

The increase in fat noted after ovariectomy complements previous experiments in which a "lipotropic effect" of estrone was demonstrated (2). In the present instance, removal of the natural source of endogenous estrogen was followed by exaggeration of the fatty acids. In rabbits kept on a normal stock diet, and not—as in our experiments—on an anti-lipotropic synthetic diet, Artom and Marziani (23) found no significant change in the total fat content of the liver following ovariectomy.

#### SUMMARY

The effect of surgical removal of various endocrine glands on liver fat was studied in rats kept on a diet low in lipotropic factors.

Thyroidectomy prevented a deposition of excess fat in the liver of rats (male and female). Adrenalectomy (in the male) and hypophy-

sectomy were accompanied by partial protection. Extreme weight loss in the latter group could have been at least partially responsible for the low liver fat. Castration in the male had no definite effect. Ovariectomy resulted in augmentation of the fat content.

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## **Inhibitory Effect of Pure and Semipurified Proteins on the Activity of Hog Kidney Conjugase**

Sirs:

In our laboratory, attempts to determine pteroylheptaglutamic acid (folic acid conjugate) in milk by use of hog kidney conjugase have always indicated that a small and rather insignificant amount of this compound was present. Tests for inhibitors (1) were made by increasing the quantity of enzyme preparation as much as 10-fold. Although the original quantity of enzyme proved to be highly active in liberating pteroylglutamic acid from pteroylheptaglutamic acid in dried brewers' yeast, in the treatment of milk samples the increased quantity of enzyme gave only very slight increases in the pteroylglutamic acid content as determined with *S. faecalis*. These results suggested that either little or no pteroylheptagultamic acid was present, or that an excessive amount of inhibitor was present in milk. Enzymatic treatment of mixtures of milk and yeast show that the presence of milk greatly inhibits the liberation of pteroylglutamic acid from the pteroylheptaglutamic acid of yeast. Therefore, milk must contain an excessive amount of inhibitor. Since hog kidney conjugase is a peptidase, and since milk protein contains a great many peptide linkages, the possibility that protein is at least partially responsible for the conjugase inhibition has been tested. The hog kidney conjugase used in these tests was prepared according to the directions given by Bird *et al.* (1), and maintained in a frozen state until used. The pteroyltriglutamic acid was a synthetic product obtained from Dr. E. L. R. Stokstad of Lederle Laboratories. The incubations were carried out at 45°C. for one hour at pH 4.5. Pteroylglutamic was determined by microbiological assay with *S. faecalis*. The results of the test are compiled in table form. These results show that protein has an inhibitory effect on hog kidney conjugase. This inhibition may be explained if hog kidney conjugase is not a specific peptidase for pteroylheptaglutamic acid and pteroyltriglutamic acid, but has a more general activity on peptide linkages.



However, this supposition has not yet been proven. If free pteroylglutamic acid is incubated with milk or with pure or semipurified proteins under the conditions described above, and the mixtures assayed directly in the presence of the protein, good recoveries are shown. However, if the protein is removed by precipitation, large losses of pteroylglutamic acid may sometimes occur, and it is for this reason

TABLE I

*Inhibitory Effect of Proteins on the Activity of Hog Kidney Conjugase*

Substrate	G. yeast incubated	G. non-yeast protein added	Ml. enzyme preparation added	Millimicrograms pteroylglutamic acid liberated
Yeast	.01	0.0	0.5	144.6
Yeast + casein	.01	1.0	0.5	31.7
Yeast + gelatin	.01	1.0	0.5	0.3
Yeast + egg albumin (commercial scales)	.01	1.0	0.5	15.9
Yeast + crystalline bovine plasma albumin	.01	1.0	0.5	2.9
Yeast + bovine plasma protein fraction V	.01	1.0	0.5	2.6
Pteroyltriglutamic acid		0.0	0.5	70.6
Pteroyltriglutamic acid + casein		1.0	0.5	42.2
Pteroyltriglutamic acid + gelatin		1.0	0.5	13.5
Pteroyltriglutamic acid + egg albumin		1.0	0.5	24.1

that the incubation of the sample with the conjugase and the subsequent microbiological assay must be carried out in the presence of protein in the case of milk and possibly certain other foods. At least, this procedure must be used until a satisfactory and convenient method of removing the protein without loss of pteroylglutamic acid is developed. It appears that hog kidney conjugase has little value in the determination of pteroylheptaglutamic acid or pteroyltriglutamic acid if only a small quantity of these compounds is present, and it is necessary to incubate the enzyme with a sample containing a large quantity of protein. The data suggest that proteins, such as gelatin, which are dispersed throughout the media may be more inhibitory than

others which are less readily dissolved or dispersed under the conditions of test.

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Received October 13, 1947

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### An Alternate Step for the Isolation of Subtilin

Sirs:

Dimick *et al.* (1) have recently described, in abstract form, an improved method for the isolation of the polypeptidic antibiotic subtilin. Details of this method will be reported elsewhere. Two important steps of the method described in the abstract (1) are (a) the extraction of subtilin from the culture at pH 2-2.5 with one-half volume of normal butanol, and (b) transfer of the subtilin from the butanol phase to an aqueous acetic acid phase by addition of petroleum ether. Whereas these steps are quite satisfactory from the standpoint of yield and manipulation on a small scale, consultation with people interested in producing subtilin on a large scale emphasized the importance of avoiding the hazard introduced by the use of highly inflammable petroleum ether. This note concerns a salting-out method of removing subtilin from the butanol phase.

The salting-out method for separating subtilin from the butanol extract is based on the insolubility of subtilin in both aqueous salt solution (2, 3) and dry butanol (1). Addition of salt to the butanol extract (pH 2) does not give satisfactory yields of subtilin, although considerable water is thereby salted out of the butanol phase. On the other hand, saturation with salt at pH 5 gives excellent yields of subtilin from the butanol (see table). The following procedure is suitable for separating subtilin from the butanol extract of cultures produced on media now in use (4). On the other hand, detailed studies of the several obvious variables would doubtless yield improved procedures.

Excess solid salt (60 g. of NaCl/l.) is added to the butanol extract with stirring. The pH of the aqueous phase is adjusted to 5 by adding 1 N NaOH to the whole mixture. The mixture is stirred vigorously for

two hours and the precipitate, which tends to collect between the two liquid phases, is separated by centrifugation in a Sharples<sup>1</sup> centrifuge (Type M-T-12-16-26 AY, fitted with a No. 34 ring dam). The precipitate collects in the bowl and the two liquid phases come off separately. In our hands, centrifugation has been an easier means of separating the precipitate from the liquid phases than filtration. The crude subtilin precipitate is washed with 10% NaCl solution, which dissolves any

*Recovery of Subtilin from Butanol by Salting-Out at pH 5*

Experiment	Recovery of subtilin activity in relation to original culture				Final product after salt fractionation (1)	
	Butanol extract <sup>1</sup>	"Salted-out" phases			Yield	Potency
		Butanol	Aqueous	Solid		
205-1	%	%	%	%	%	—
213 and 214-1	81	2	—	63	—	—
215	101	3	<0.3	90	—	—
216 and 217	85	3	<0.1	85	59	229
	90	4	<0.1	67	—	211

<sup>1</sup> The cultures in these experiments had from 620 to 880 units of subtilin per liter (i.e., they had activity corresponding to 620 to 880 mg. of our arbitrary subtilin standard (L1263), which is about half as active as the most potent subtilin yet obtained).

solid NaCl that may be present, and is then extracted with absolute ethanol. On a salt-free basis this precipitate has a potency within 10–20% of the most potent final product so far obtained. Subsequent purification, which removes toxic materials, is accomplished by salt fractionation as described by Dimick *et al.* (1). The accompanying table shows that good yields are obtained by the salting-out procedure. However, an unaccounted-for loss may occur in the salting-out step. The final yields and the final potencies are as high as any yet obtained.

We are indebted to E. M. Humphreys and P. A. Thompson for microbiological assays.

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Received October 27, 1947

HANS LINEWEAVER  
A. A. KLOSE  
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<sup>1</sup> The mention of this product does not imply that it is endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

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### Scorbutic Symptoms in Vitamin A-Deficient Rats <sup>1</sup>

Sirs:

In the course of an investigation on the vitamin A-deficiency syndrome in rats, it was observed that certain of the symptoms resembled those of acute scurvy. These symptoms were: bleeding of the lachrymal glands, red, swollen gums with evidence of bleeding, and an early paralysis of the forelegs, with swelling of the joints. The contention that these symptoms were scorbutic in nature was supported by the fact that a marked and rapid improvement in the condition of the animals, particularly in reference to bleeding and paralysis, was obtained after intraperitoneal injection of massive doses (50 mg.) of vitamin C. Food consumption was temporarily increased, perhaps because of an improved buccal condition. That such effects are not totally unexpected is evident from earlier suggestions (1, 2, 3, 4) that vitamin A-deficient rats exhibit lowered blood and tissue levels of vitamin C.

Blood ascorbic acid levels of the vitamin A-deficient animals were always lower (50–66%) than the controls. This is in agreement with an earlier observation (3).

More remarkable still were the differences observed in the adrenals: the ratio of adrenal weights to body weight was from 2 to 4 times larger for the deficient animals than for the controls; despite the hypertrophy, the total adrenal ascorbic acid was consistently lower than that of the controls. Adrenal hemorrhage was evident in addition to hypertrophy. The development of the marked scorbutic symptoms reported here in vitamin A-deficient rats might be related in part to the fact that an essentially synthetic diet (sucrose, casein, corn oil, mineral salts, and crystalline vitamins) was used. In previous vitamin

<sup>1</sup> The authors are indebted to the National Vitamin Foundation, 150 Broadway, New York, New York, for a grant in support of this work.

A-deficient diets, yeast, rather than crystalline vitamins, was used. It must not be construed in the present work that "true" vitamin A deficiency symptoms were alleviated; only the conditions associated with vitamin C deficiency were improved.

The nature of this apparent relationship between vitamin A, vitamin C, and the adrenal cortex is being investigated.

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## Book Reviews

**Le pH et sa mesure. Les potentiels d'oxydo-reduction, le rH.** By M. HUYBRECHTS, Professor at the University of Liège. 4th ed. Masson & Cie, Paris, 1947. 467 pages. Price 400 fr.

This is the fourth edition of a book published for the first time in 1932. The preface to the fourth edition is dated 1945. Considering the state of scientific work in Europe in the occupied countries during this period, this is an unusual success and shows the usefulness of the book more than any favorable review might be able to do. The book is very elementary, probably the most elementary ever written on the subject. It begins with a possibly elementary introduction into such chapters of physical chemistry as are necessary for the understanding of the concepts and methods involved in the determination of pH and redox potentials. There is even an appendix dealing with the most elementary principles of algebra. Such a textbook will be very useful for those requiring tuition really *ab ovo*.

The book is divided into two parts. The first deals with various chapters of physical chemistry, such as the mass action law, osmotic pressure, electrolytic dissociation, gas laws, galvanic cells in general, hydrogen electrodes, and other methods for the determination of pH. The second chapter deals with the theory of reversible oxidation-reduction. The book is, however, not intended as a manual for use in the laboratory.

L. MICHAELIS, New York, N. Y.

**Recent Advances in Clinical Pathology.** By various authors, produced under the auspices of the European Association of Clinical Pathologists. The Blakiston Co., Philadelphia, Penna., 1947. 468 pp. Price \$5.50.

This book contains sections on bacteriology, biochemistry, hematology and cytology, and histology. The articles of the book are written by authors with special experience in these particular fields. The chapter titles are self-explanatory. This book is intended to help the clinical laboratories in their daily routine work. It describes the various techniques used in different laboratories and experimental laboratories. The section of histology, edited by A. H. T. Robb-Smith, Oxford, deserves special praise. It is an excellent critical review of all the newer techniques in biopsy as applied to various human organs. Good histological photographs illustrate the text.

The section on biochemistry is apparently written for the purpose of supplying the clinicians with some critical knowledge in evaluation of selected chemical function tests. The sections on bacteriology and hematology are comprehensive and make this book a valuable reference in the routine of technical laboratories of a modern hospital.

S. J. THANNHAUSER, Boston, Massachusetts.

**Viruses.** By MAX A. LAUFFER, Associate Research Professor of Physics in the University of Pittsburgh. From the Twentieth Annual Priestley Lectures, The Pennsylvania State College, 1946. Litho-printed by Edwards Bros., 62 pp., 42 figs. Price \$2.00.

The first chapter, on viruses as molecules, opens with a short description of Iwanowsky's original discovery and its significance. The protein nature of viruses is outlined and the 15-17 common amino acids which have been isolated or identified from tobacco mosaic virus are given. The author points out the interesting fact that, in the interior of the plant cell, the virus crystallizes as hexagonal plates instead of the long slender needles obtained in the beaker. The evidence familiar to plant virus workers and now accepted by them, which equates the high molecular weight protein with the virus itself is outlined. Some of the mutations of the tobacco mosaic virus are described and the variations in their amino acid content tabulated.

The author deals briefly with the unanswerable question, so frequently asked by the layman, "are viruses living things?" He states the two extreme points of view, one that viruses are chemical compounds with an ability to reproduce by an autocatalytic synthesis from raw materials present in the host cell, and the other that viruses are merely highly specialized living organisms whose material substances exhibit a few unusual chemical and physical properties or, as Laidlaw put it, perfect parasites living a borrowed life.

Does the hypothesis that a virus is a protein molecule actually fit all the facts? To fulfill the definition of a molecule, the virus particle should be incapable of subdivision without change in the chemical and biochemical properties and should be of the same size and shape. This definition cannot yet be applied to the particles of tobacco mosaic virus since we are not sure what is the exact particle size of this virus. The situation is different for some of the spherical viruses such as that of tomato bushy stunt. In this case it has been possible to show by means of the ultracentrifuge that the virus particles are identical in size and shape. However, even if we cannot yet be certain that some viruses are molecules it is true to say that much of our knowledge of the nature of viruses is due to chemical and physical techniques based on this conception. A number of viruses have now been isolated in crystalline form and several have been photographed on the electron microscope. Some excellent illustrations of virus crystals and electron micrographs of virus particles are given.

In Chapter 2 the various technical methods for measuring the size and shape of the virus particle are discussed, the electron microscope, the ultracentrifuge, viscosity measurements, *etc.* The results of all these methods as applied to two viruses, those of tobacco mosaic and influenza A, are dealt with in some detail and, when tabulated, it can be seen that all the methods give essentially the same value for the length and thickness of the tobacco mosaic virus particles. So far as influenza A is concerned, the particles do not seem to be strictly uniform in size.

The question of the hydration of virus particles and its bearing on particle size are also dealt with.

In Chapter 3 the inactivation and denaturation of virus proteins are discussed, with particular reference to the tobacco mosaic and influenza viruses. Three methods of distintegration of tobacco mosaic virus are described; by heat, by high pressure and by the action of urea. In considering the inactivation of influenza A virus three

biological activities are envisaged; first is the ability to infect animals, including man, the mouse and the chick embryo. Another property of the virus is its ability to agglutinate red blood cells of chickens, and a third biological activity is the ability to induce specific protecting antibodies in the blood stream of the host. It is found that the reaction velocity for the destruction of hemagglutinin increases as one decreases the concentration of virus. This question of the destruction of influenza A virus hemagglutinin has its practical aspect. When the rate of destruction of hemagglutinin is studied, clues might be provided for establishing conditions most favorable for the storage of influenza vaccine.

In Chapter 4 the author discusses viruses as organisms and gives some data on the rate of multiplication of viruses. For tobacco mosaic virus he calculates that from  $10^{-11}$  g. of virus placed on a leaf,  $10^{-1}$  g. of virus can be recovered. This represents a multiplication of 10 billion-fold. Similar or greater multiplication rates can be postulated for tobacco necrosis and influenza viruses. Such rates of multiplication are apparently compatible both with the theory of particle division and that of an autocatalytic reaction. Another property of viruses in common with living organisms is that of the power to mutate, and the author states that many viruses can be modified by passing them through unnatural hosts. Whether this is true of plant viruses is a moot point; an alternative hypothesis is a selection of existing strains rather than a modification of a single virus.

The theories of virus infectivity are next dealt with and the evidence is seen to be greatly in favor of the view that virus infection is the result of at least one virus particle being present in a favorable location.

In the last chapter Dr. Lauffer discusses viruses and human welfare and comments upon some of the great epidemics and pandemics of past years. So far as the control of plant virus diseases is concerned the best hope seems to lie with the plant breeder. We do not, however, quite agree with the statement that virus X does not adversely affect the potato plant. On the contrary, this virus, even when carried without symptoms, may reduce the yield by as much as 10%.

As regards the control of virus diseases of animals and man, there seem to be two general types of approach. First the prevention of the spread of the causative agent, either by the suppression of the insect vector, and here some of the new insecticides may play an important part, or by prevention of contact. The second approach consists of the modification of the host by either active or passive immunization. The most striking example of this method is the vaccine now in use against yellow fever; millions of people have been successfully inoculated against this dread disease. A vaccine is also apparently available against dengue or "break-bone" fever.

The author states that influenza and measles are among the virus diseases which have gone down in defeat before the forces of science. We hope he is right.

When we consider the great importance of viruses in man's economy and the fearful threat some of them are to his well-being, it is sad to learn of the imminent closing of the Rockefeller Institute at Princeton where so much good work on animal and plant viruses has been carried out. In England at the present moment the worst epidemic of poliomyelitis for years is raging and the total annual sum spent on the investigation of this virus disease is about £2,000. The total annual sum spent on war research is in the neighborhood of £60 millions. Truly knowledge comes but wisdom tarries.



Dr. Lauffer is to be congratulated on his Priestley Lectures which, while being guided to some extent by his own studies, yet give a clear and concise account of some important aspects of the virus problem.

KENNETH M. SMITH, Cambridge, England.

**Fatty Acids, Their Chemistry and Physical Properties.** By KLARE S. MARKLEY, Principal Chemist, Oil, Fat, and Protein Division, Southern Regional Research Laboratory, U. S. Department of Agriculture, New Orleans, Louisiana. Interscience Publishers, Inc., New York, N. Y., 1947. 668 pp. Price \$10.00.

The author's purpose in writing this volume was "to bring together in an organized and readily accessible form as much as possible of the present accumulation of facts and data pertaining to the chemical reactions and physical properties of the fatty acids and, especially, of the long chain fatty acids which comprise the building stones of all natural fats, oils, and waxes."

No other volume has dealt exclusively with the fatty acids *per se*. The author has pioneered in reviewing the published literature on fatty acid chemistry extending from about 1815 to the early part of 1945. He has endeavored to include only the most reliable information and has weeded out much that was based on the study of obviously impure materials. As a result only about 1500 authors have achieved listing in the author index. The test is documented with approximately 1500 literature references, few of which are duplicates. This feature of the volume would bear amplification, for there are numerous instances in which it is difficult to determine original sources of data. The coverage of fatty acids from fats and oils is excellent, but some pertinent material on the fatty acids from waxes appears to have been missed.

The volume is logically subdivided into six main sections.

- A. The Nature and History of Fats and Waxes.
- B. Classification and Structure of the Fatty Acids.
- C. Physical Properties of the Fatty Acids.
- D. Chemical Reactions of the Fatty Acids.
- E. Synthesis of Fatty Acids.
- F. Isolation and Identification of Fatty Acids.

Sufficient introductory material is provided so that anyone fairly conversant with organic and physical chemistry should find the volume easily readable. After a brief introduction to the most important individual fatty acids, the matter of structural isomerism and stereoisomerism is considered.

The discussion on physical properties brings together much useful data. This is one of the better sections of the book. Properties in the crystalline and liquid states and in solution as well as properties dependent on molecular structure are considered.

The diversity of chemical reactions possible with fatty acids is quite amazing and this discussion should do much to intensify interest in these potentially abundant raw materials. The reactions are considered from the standpoint of each reactive center of the molecule, i.e. the hydrogen ion, the hydroxyl group and carbonyl group as well as the carboxyl group as a whole, the hydrogen of the alkyl chain and unsaturated linkages. An exhaustive review of the extensive literature on hydrogenation of unsaturated fats and fatty acids was considered beyond the scope of the book. A good coverage of the literature on oxidation at the double bond is given and serves at once

to emphasize the need for more intensive investigation and the difficulties to be encountered in this phase of fatty acid chemistry.

Some of the outlined methods of synthesis of fatty acids are of academic interest only since pure fatty acids are readily available from natural sources, but others are of real usefulness in fatty acid research. The discussion of theories of *in vivo* synthesis makes fascinating reading.

The section on isolation and identification is worthy of careful study by workers in this field. Adherence to the principles outlined would do much to improve the quality of future research.

The author's decision to emphasize the naturally occurring long-chained fatty acids stems from a practical rather than an academic interest in fatty acid chemistry. This results in certain peculiarities in the arrangement of the material. For example, physical data on the important class of dibasic acids are omitted from the section on physical properties but appear in the next following section on chemical reactions incidental to the discussion of oxidation of unsaturated acids.

The volume is well printed on excellent paper and is sturdily bound. Typographical errors are exceedingly rare. There are minor errors in rhetoric and a few obviously inconsistent statements. These do not detract seriously from the general excellence of the work. Among the inconsistencies noted is the use of both the one number and two number systems for locating double bonds. It would appear that the author missed an opportunity to come out strongly for one system or the other, preferably the simpler one number system.

This book will be an exceedingly useful reference book for persons engaged in fat and oil chemistry. It should be useful as a text for seminar courses. In addition it should be valuable to organic chemists in general in pointing out the vast opportunities for research in fatty acid chemistry.

A. W. WEITKAMP, Whiting, Indiana

X **Volumetric Analysis, Vol. II, Titration Methods.** By I. M. KOLTHOFF, Professor and Head, Division of Analytical Chemistry, Univ. of Minnesota, and V. A. STENGER, Analytical Research Chemist, The Dow Chem. Co., Midland, Michigan. Interscience Publishers, Inc., New York, 1947. xiii + 388 pp., 14 illustrations. Price \$6.00.

As stated in the Preface, the contents of this book are based upon a German monograph, "Die Praxis der Massanalyse," published in 1928 by Kolthoff with the assistance of H. Menzel of Dresden, and revised in 1931. The first edition was translated into English by N. H. Furman in 1929. This volume is the second of a three volume set. Volume I deals with the detailed theoretical treatment of the more important procedures, and, since an understanding of theoretical principles is essential in evaluating analytical methods, many back references are made to Volume I.

This authoritative treatise on volumetric analysis has not been written to cover exhaustively all applications of volumetric methods to specialized subjects but has given a somewhat arbitrary selection of useful and reliable procedures for certain purposes. The arrangement of the methods is determined by the type reaction involved in the titration, rather than by the field in which it may be applied. This reviewer was well pleased with the rather exhaustive and well written notes on applications following the procedures. Numerous charts and tables are given which the reader will find of real value.

This volume is divided into three parts. Part A deals with the calibration and use of volumetric apparatus and the practical principles of volumetric analysis. Part B with acidimetry and alkalimetry, acid-base reactions, acid-base displacement titrations, titrations involving hydrolytic precipitation or complex formation, and one chapter is devoted to special methods of acidimetry and alkalimetry, including the determination of various organic substances, such as chloral hydrate, salts of organic acids, acid anhydrides, acid amines, mercaptans, theobromine, *etc.* Part C treats of quantitative precipitation and complex-formation reactions where one whole chapter is devoted to argentometric titrations, another chapter to 7 other well chosen precipitation methods which the analyst will find very valuable in his work. The third chapter in part C deals with determination of halides of mercury with thiocyanate, of iodine with mercuric chloride, and miscellaneous methods, such as determination of platinum metals with sodium diethyldithiocarbamate.

This volume along with Volumes I and III, which is to be a treatment of oxidation-reduction methods (to be published in 1948), is a very worth-while contribution to the field of analytical chemistry, and should be included in the library of any one interested in quantitative analysis.

LEO K. YANOWSKI, New York, N. Y.

**Les cancers produits par des substances chimiques exogenes.** By ANTOINE LACASSAGNE, Professor, College of France; Director, Institute of Radium. Hermann and Co., Paris, France, 1946. 166 pp. Price 200 francs.

This is a general factual discussion, for the most part, dealing with the production of cancers by various types of exogenous carcinogenic compounds, with a fitting emphasis on the polycyclic aromatic hydrocarbons.

There are six chapters: one dealing with production of cancer by arsenic compounds, one dealing with nitrogenous carcinogens, and four dealing with the carcinogenic action of polycyclic aromatic hydrocarbons. The four latter chapters deal with (1) application to the skin, (2) injection and introduction into body cavities, (3) lung and stomach cancers, and (4) general action, lymphoid cancers and leucemias.

There are a total of about 350 references which are reasonably up-to-date. Altogether, the volume commends itself as a valuable monograph.

ROGER J. WILLIAMS, Austin, Texas

# Thermal Destruction of Influenza A Virus Infectivity<sup>1,2,3</sup>

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Received September 17, 1947

## INTRODUCTION

In previous publications, the results of studies on the thermal destruction of the red blood cell-agglutinating ability of PR8 influenza A virus were reported (1, 2, 3). These investigations showed that the destruction of the hemagglutinin is a complex process which behaves superficially like a reaction of the three-halves order. The energy of activation for the process in neutral buffers was found to be about 110,000 cal./mole. In view of the foregoing, it was thought worthwhile to investigate the destruction of the infectivity of influenza A virus at high temperatures, in order to determine wherein this process differs from the destruction of the hemagglutinin.

## MATERIALS AND METHODS

### *Virus Preparation A*

Fertile White Leghorn eggs were incubated for 10 days at 39°C. and were then inoculated with diluted stock inoculum which consisted of allantoic fluid from chicken embryos infected with the PR8 strain of influenza A virus. The history of the virus and the nature of the stock inoculum were described previously (1). After incubation for 40–48 hrs. at 37°C., the inoculated embryos were chilled for 24 hours at 4°C. The allantoic fluids were then harvested and pooled. After several liters had been collected, the virus was concentrated by centrifugation in a Sharples Supercentrifuge according to the method described by Stanley (4). The sedimented virus was suspended in 0.1 *M* phosphate buffer at pH 7. Nitrogen determinations made by the Kjeldahl method yielded a value of 0.4 mg. of nitrogen/ml., corresponding to about 4 mg. of virus/ml.

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<sup>1</sup> Aided by a grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

<sup>2</sup> Aided by a grant from the National Foundation for Infantile Paralysis.

<sup>3</sup> Contribution No. 8p-47 of the Department of Physics of the University of Pittsburgh.

*Virus Preparation B*

Phenylmercuric nitrate at a concentration of  $0.5 \times 10^{-5}$  g./ml. was added to a pooled batch of allantoic fluid from chicken embryos infected with stock inoculum of PR8 influenza A virus. The pH of the material was 7.4.

*Virus Preparation C*

Preparation B was passed through two generations in chicken embryos. The allantoic fluid from the second passage was preserved with phenylmercuric nitrate at a concentration of  $0.5 \times 10^{-5}$  g./ml.

*Virus Preparation D*

Preparation C at a dilution of  $10^{-4}$  was inoculated into embryos. The fluid was harvested after 48 hours of incubation and was then pooled. No preservative was added.

*Infectivity Measurements*

Decimal dilutions of virus samples to be titrated were made with 0.1 *M* potassium phosphate buffer at pH 7. Five embryos were inoculated with each of 6 dilutions covering the expected endpoint range. The dose in each case was 0.2 ml. The inoculated embryos were incubated for 48 hr. at 37°C. and then chilled and harvested. The fluid of each was tested for ability to agglutinate chicken red blood cells. A positive test was regarded as evidence of infection of the embryo. Fifty per cent infectivity endpoints were calculated by an algebraic variation of the method of Reed and Muench (5). The per cent positive and per cent negative responses were accumulated in the usual manner, and the 50% endpoint was regarded as that concentration at which the accumulated positive and the accumulated negative responses were equal. Usually it was found to lie between two of the concentrations tested. The position of the endpoint was expressed as a decimal fraction of the logarithmic interval between the more concentrated and the more dilute virus solutions giving responses just greater than and just less than 50%, respectively. It can be shown that this decimal fraction is equal to  $\frac{(P_1 - N_1)}{(P_1 - N_1) + (N_2 - P_2)}$ , where  $P_1$  is the accumulated positive score and  $N_1$  the accumulated negative score at the higher concentration and  $P_2$  and  $N_2$  are the corresponding quantities at the lower concentration. This algebraic process is simpler than the graphic method described by Reed and Muench for determining the decimal fraction in question, and it yields exactly the same result.

## PRESENTATION AND DISCUSSION OF EXPERIMENTAL RESULTS

*Order of the Reaction*

Small test tubes, each containing 0.1 ml. of virus preparation A, were heated for 5, 30, and 60 minutes, respectively, at 45°C. A similar sample held at room temperature served as a control. Fifty per cent infectivity endpoints were determined for

each. Similar data were obtained at 56°C. A comparable test was carried out at 48°C. on virus preparation D diluted with an equal volume of potassium phosphate buffer at pH 7. The results are shown in Fig. 1, where the  $\log_{10}$  of relative activity is plotted

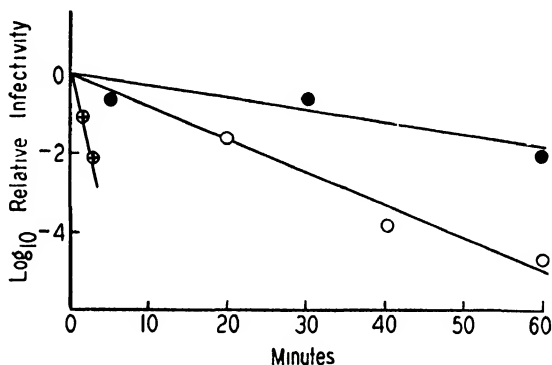


FIG. 1. Infectivity of PR8 influenza A virus after various times of heating, plotted according to the law of a first order reaction.

●—Virus preparation A, 45°C.; ○—Virus preparation D + phosphate buffer at pH 7, 48°C.; ⊕—Virus preparation A, 56°C.

against time. It can be seen that the data fit straight lines within the limits of the error discussed in a subsequent section. These data are consistent with the assumption that the thermal destruction of embryo infectivity is a reaction of the first order.

### *Temperature Coefficient*

The specific reaction rates for thermal inactivation of influenza A virus are equivalent to 2.3 times the slopes of the straight lines presented in Fig. 1. On the assumption that the reaction is of the first order, specific reaction rates for different conditions can be calculated when the loss in infectivity after a 10 minute period of heating is known. Numerous estimates of the specific reaction velocity constant at various temperatures were obtained in this manner for the destruction of the infectivity of preparation A and B in 0.1 *M* phosphate buffer at pH 7. The constants so obtained, and those calculated from Fig. 1, are presented in Table I.

For most chemical reactions, when natural logarithms of reaction velocity constants are plotted against reciprocals of absolute temperatures, straight lines are obtained. In Fig. 2, the data of Table I are plotted in that manner. If allowance is made for considerable error, a straight line can be fitted to these data. If the data are extrapolated to

TABLE I

*Specific Reaction Velocity Constants at Various Temperatures for the Destruction of PR8 Influenza A Virus Infectivity*

Temperature in °C.	$k$ in min. <sup>-1</sup> Preparation A	$k$ in min. <sup>-1</sup> Preparation B
56	1.84, 1.26	—
55	0.92	—
54	1.38	1.00
52	0.92	1.34
50	0.46, 0.46 0.54, 0.55	—
48	—	0.72
46	—	0.54
45	0.11, 0.15 0.07	—
44	—	0.37, 0.41
42	—	0.17
40	—	0.13

4°C., a value for  $k$  of about  $10^{-4}$  min.<sup>-1</sup> is obtained. This would correspond to a decrease of activity of about two logarithmic units in a month. This is in satisfactory agreement with the result obtained by Miller (6) for PR8 virus in pH 7 phosphate buffer at 4°C.

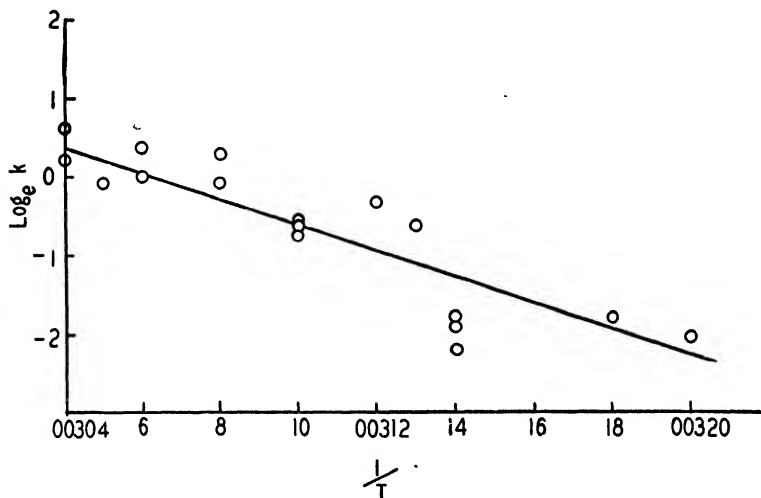


FIG. 2. The relationship between the specific reaction rate for the destruction of PR8 influenza A virus infectivity and absolute temperature, plotted according to the Arrhenius equation.

*Energy and Entropy of Activation*

According to the theory of absolute reaction rates (7), the specific reaction rate is related to the absolute temperature by the following equation:

$$\log_e k = \log_e \frac{RT}{Nh} + \frac{\Delta S^*}{R} - \frac{\Delta H^*}{R} \frac{1}{T}.$$

$R$  is the gas constant,  $T$  the absolute temperature,  $k$  the specific reaction velocity constant,  $N$  Avagadro's number,  $h$  Planck's constant,  $\Delta S^*$

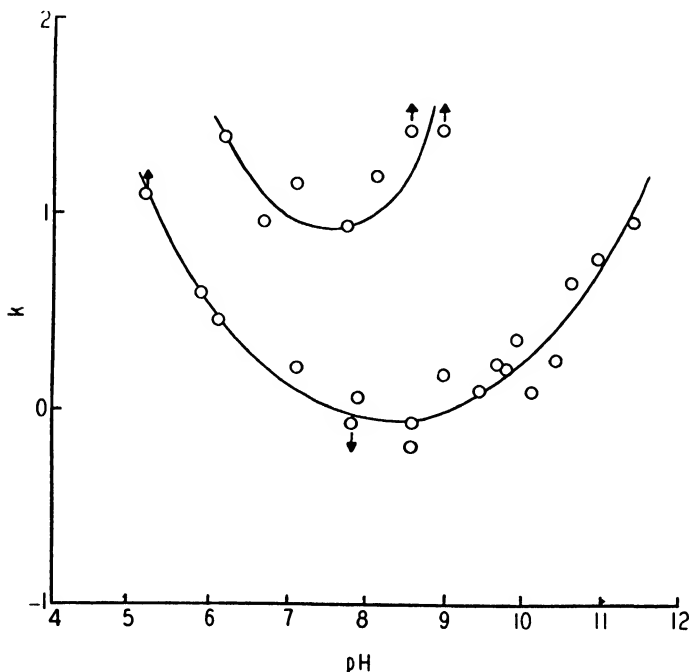


FIG. 3. The relationship between the specific reaction rate for the destruction of PR8 influenza A virus infectivity and pH. Upper curve, 54°C., lower curve, 46°C.

the entropy of activation, and  $\Delta H^*$  the energy of activation. The energy of activation divided by the gas constant is approximately equal to the slope of the line relating to  $\log_e k$  to the reciprocal of absolute temperature, as illustrated in Fig. 2. After the energy of activation has been evaluated, the entropy can be calculated by means of the above equation. From the data presented in Fig. 2, the energy of activation



was calculated to be  $34,000 \text{ cal. mole}^{-1}$ , and the entropy of activation was calculated to be  $39 \text{ cal. mole}^{-1} \text{ deg.}^{-1}$ .

### *Effect of pH*

Equal portions of virus preparation C and various buffers of ionic strength 0.2 at different pH values were mixed and immediately heated for 10 minutes at either  $46^\circ$  or  $54^\circ\text{C.}$ , and then cooled at once. pH measurements were made on each mixture with a Beckman pH meter. Fifty per cent chicken embryo infectivity end points were determined for each heated sample. Numerous determinations of the endpoint for the unheated virus were carried out. The rate of inactivation in reciprocal minutes at each pH value was determined by subtracting the negative logarithm of the endpoint from the average of the negative logarithms of the endpoints of the unheated virus, by multiplying by 2.3, and by dividing by 10. The results are presented in Fig. 3. It can be seen that the destruction of infectivity proceeds at a minimum rate at a pH of about 8.5 at  $46^\circ\text{C.}$  and at about 7.5 at  $54^\circ\text{C.}$  This means that the infectious principle has maximum stability at pH values near 8. It was found previously that the red blood cell-agglutinating activity has a maximum stability at  $55^\circ\text{C.}$  at pH 8.4 (2).

### *Reproducibility of Experimental Data*

Nine replicate titrations on virus preparation C and 6 replicate titrations on virus preparation D were made. The negative logarithms of the 50% endpoints are recorded in Table II. The figures represent

TABLE II  
*Negative Logarithms of 50% Infectivity Endpoints of Untreated Virus*

Preparation C		Preparation D
5.50*		8.28
7.00		8.80
6.50		8.62
7.60		8.45
6.62		8.11
6.57		8.43
7.33		
6.88		
7.36		
<hr/>		<hr/>
6.9825	Mean	8.4483

\* This value was omitted when the mean and the standard deviation were computed. It differs from the mean for preparation C by 1.48 units. Since the best estimate of the standard deviation for  $-\log_{10}$  50% endpoints is 0.35 unit, the value 1.48 is 4.2 standard deviation units from the mean. The probability that such a difference could have arisen by random errors is about one in a thousand. It is, therefore, reasonable to omit it when computing statistics.

estimates of the concentrations of original fluid which will cause infection in 50% of the embryos inoculated with 0.2 ml. of inoculum. From the data in Table II, one can calculate that the mean value of the negative logarithm of the titer is 6.98 for preparation C and 8.45 for preparation D. The standard deviation for the combined data representing preparations C and D was calculated in the manner described by Lauffer and Miller (8) to be 0.35 unit. This value is considerably greater than that obtained by Knight (9) for the titration of influenza virus with chicken embryos when 10 embryos were used per dilution. It is also somewhat, though probably not significantly, greater than the variation found by Knight for studies carried out using 5 embryos per dilution.

It is possible to obtain an estimate of the standard error of the reaction velocity constants presented graphically in Fig. 3. These constants were all obtained by multiplying by 2.3/10 the difference between the mean negative logarithmic endpoint of preparation C and a single negative logarithmic endpoint on the material after 10 minutes of heating. The standard error of the mean for sample C was calculated to be 0.124. The standard error of the single determination after treatment must be assumed to be the same as the value obtained for the standard deviation of the distribution of negative logarithmic endpoints. The standard error of the difference between the mean for the sample and a single determination after treatment was calculated to be 0.37.

The standard error of the rate constant is obviously equal to the standard error of the difference multiplied by 2.3/10 or 0.085. This is considerably less than 10% of the highest rate constants presented graphically in Fig. 3, but it is of the same order of magnitude as some of the lower rate constants. However, it is clear that the difference between the rate constants at the extreme values of pH and those at intermediate values greatly exceeds the standard error of the determination.

#### *Relation to Destruction of Red Blood Cell-Agglutinating Activity*

The energy and entropy of activation for the destruction of red blood cell-agglutinating activity of PR8 influenza A virus in 0.1 M phosphate buffer at pH 7 were found by Lauffer and Scott (2) to be 110,000 cal. mol.<sup>-1</sup> and 300.8 cal. mol.<sup>-1</sup> deg.<sup>-1</sup> respectively. By substitution of these values in the equation representing the theory of absolute reaction rates, one can show that the destruction of chicken red blood cell-agglutinating activity should proceed very slowly in the

temperature range in which infectivity decreases at appreciable rates. Direct experiments tend to bear out this prediction. In 3 experiments on preparation B, it was found that the red blood cell-agglutinating activity was practically unchanged after heating for 10 minutes at temperatures below 56°C., yet the infectivity was reduced greatly by heating for 10 minutes at temperatures as low as 46°C. It can be concluded with complete assurance that infectivity destruction proceeds much more rapidly than the destruction of agglutinating activity. Even though, as was shown previously, the pH of maximum stability of the hemagglutinin and of infectivity are similar, this result demonstrates clearly that the destruction of activity and that of hemagglutinin are completely independent reactions, possibly involving different centers in the virus particle.

### SUMMARY

The destruction of the infectivity of PR8 influenza A virus seems to proceed as a reaction of the first order. It was calculated that the energy of activation has a value of about 34,000 calories per mole, and that the entropy of activation, calculated according to the theory of absolute reaction rates, has a value of about 39 calories per mole per degree. The destruction of infectivity was found to be much more rapid than the destruction of red blood cell-agglutinating activity. Maximum stability of the infectivity occurred at pH values between 7.5 and 8.5. The standard deviation of the distribution of negative logarithmic 50% infectivity end points was found to be 0.35.

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## Preliminary Observations on the Adsorption of Prothrombin by Barium Sulfate

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Received June 23, 1947

### INTRODUCTION

Bordet and Delange in 1912 (1) first reported that barium sulfate had the ability to adsorb some substance from oxalated plasma, thus preventing subsequent clotting on the addition of calcium and tissue juice, but not preventing clotting on the addition of thrombin. They termed the adsorbed substance serozyme, assuming it to have some enzymatic activity. Dale and Walpole in 1916 (2) repeated these experiments and further clarified the reaction in more modern terms. Dale and Walpole utilized the effect of barium sulfate on plasma to obtain a prothrombin-free source of fibrinogen. In 1946 Tanturi and Banfi (3) used the prothrombin-free plasma from this reaction to study the mechanics of the one-stage test for prothrombin concentration; serial dilutions of normal plasma were made by using identical plasma treated with barium sulfate as a diluent, and a prothrombin concentration curve was plotted from the prothrombin times. Quick (4) used the same procedure in first devising his technique for prothrombin concentration in 1937, but used  $\text{Al}(\text{OH})_3$  gel, instead of barium sulfate, to remove prothrombin from the diluent plasma. In the past year, Rosenfield and Tuft (5) adapted the Quick curve by using the more efficient barium sulfate adsorbent.

On addition of about 10% U.S.P. or C.P. powdered barium sulfate to oxalated plasma, and incubation of the mixture at 37°C. for 10 minutes, prothrombin is completely removed from the plasma by adsorption on the barium sulfate. The mixture is then centrifuged at 1,500–3,000 r.p.m. for from 10 to 30 minutes. The clear supernatant prothrombin-free plasma is readily decanted from the barium sulfate sediment. This prothrombin-free plasma fails to clot when calcium ion and thromboplastin are added optimally and the mixture incubated at 37°C. Clotting does take place, within 3 seconds, upon the addition of strong bovine thrombin. Rosenfield and Tuft were unable to discover any difference between the amounts of fibrin formed in barium sulfate-treated and -untreated oxalated plasma.

Because barium sulfate apparently removes all prothrombin, barium sulfate after adsorption of prothrombin was carefully washed repeatedly with saline and digested for nitrogen by simple micro Kjeldahl technique.

The procedure for this was as follows: In a 50 cc. calibrated Pyrex centrifuge tube, 1.00 cc. of oxalated plasma was mixed with 0.2 cc. of a 30% suspension of powdered C.P. barium sulfate in water. The mixture was incubated in a 37°C. water bath for 10 minutes and then centrifuged at 2,000 r.p.m. for 20 minutes. The supernatant plasma was discarded and the sediment was thoroughly mixed with about 35 cc. of saline and again centrifuged in a similar manner. The saline was discarded and fresh saline washing was repeated once again. More than two washings were found to be unnecessary. The packed and twice washed sediment was then dispersed in 2 cc. of sulfuric-phosphoric acid digestion mixture, and heated by micro burner until all color was dissipated. A drop or two of 8% hydrogen peroxide was always added to complete the digestion. At the end of the digestion, water was added to the 35 cc. volume mark, and, after thorough mixing, the tube was again centrifuged at 2,000 r.p.m. for 20 minutes. Seven cc. of the supernatant fluid was then taken off and mixed with 3 cc. of Nessler's solution. The resulting color was read in a model 11 Coleman spectrophotometer set at 420  $\lambda$  (the instrument had been previously calibrated against known nitrogen standards). Since exactly 1 cc. of plasma was used as a source of nitrogen for the barium sulfate digestion, the results for convenience are reported ( $\times 100$ ) in terms of mg./100 cc. of plasma. For further exactness, this figure must be corrected for whatever dilution occurred at the time of original oxalation of the blood; e.g., 9.0 cc. whole blood plus 1.0 cc. of 0.1 *M* oxalate ( $\times 10/9$ ). Duplicate determinations always agreed within 5%.

The result of 17 such studies on both normal and abnormal bloods are shown in Table I and are compared with the prothrombin activity of each blood. The prothrombin activity studies were made by the authors' modification of the Quick method (5).

Normal bloods (Table I) yielded 13.4–19.4 mg.-% nitrogen upon digestion of the barium sulfate adsorbent. Using the usual 6.25 nitrogen factor for conversion to protein, a value of from 84–121 mg.-% protein is obtained. This value differs from the value of 20 mg.-% reported by Seegers *et al.* (6) to be the probable prothrombin level in human blood, but barium sulfate is very likely to adsorb other nitrogenous material.

Barium sulfate was used to adsorb prothrombin from plasmas with diminished prothrombin activity. After repeated washing, this barium sulfate was digested in a similar manner in an attempt to transpose the difference in prothrombin activity to terms of mg. of nitrogen. However, completely paradoxical results were obtained (Table I). It appears that the barium sulfate adsorbs nitrogenous material in an inverse proportion to the prothrombin concentration. Although this

TABLE I

*Adsorption of Prothrombin by Barium Sulfate; Comparison of the Nitrogen Adsorbed to Barium Sulfate (Determined by Micro Kjeldahl Analysis) and the Prothrombin Concentration in Normal and Abnormal Plasmas*

Case no.	Diagnosis	10% Dln. proth. time	Proth. activity 13.4 ( $\frac{\text{time}-10.8}{\times 100}$ )	mg.-% Adsorbed N	(N $\times$ 6.25) Adsorbed prot.
		(sec.)			
1	Normal	23.6	100%	14.6	91.6
2	Normal	23.2	100%	15.3	95.8
3	Normal	20.7	100%	15.6	97.3
4	Normal	23.7	100%	13.4	84.1
5	Normal	24.8	96%	15.0	93.8
6	Normal	22.4	100%	19.4	122.0
7	Acute rheumatic fever with H. D. (no salicylates)	23.5	100%	15.6	97.3
8	Bilateral femoral thrombophlebitis	23.9	100%	14.3	89.6
9	Biliary cirrhosis	52.0	33%	25.1	157.0
10	Case No. 9 after Vitamin K therapy	21.1	100%	16.5	103.0
11	Acute rheumatic fever on salicylates	79.5	19%	22.9	143.0
12	Acute coronary thrombosis (died 36 hrs.)	25.9	89%	14.9	93.2
13	Pulmonary emboli (femoral) on dicoumarol	49.7	34%	18.7	117.0
14	Banti syndrome (cause unknown)	26.8	84%	20.2	126.0
15	Generalized carcinomatosis (gastric)	29.1	73%	22.4	140.0
16	Acute myocardial infarction on dicoumarol	28.3	77%	19.7	123.0
17	Late post-operative phlebitis on dicoumarol	69.7	23%	20.2	126.0

relationship is not linear, the highest nitrogen values are correlated with the lowest prothrombin values. The significance of this observation is not clear at present.

### SUMMARY

1. Micro Kjeldahl digestion of barium sulfate used to adsorb prothrombin from oxalated plasma shows about 13.4–19.4 mg.-% nitrogenous material removed from plasma so treated.

2. When prothrombin activity of blood is decreased, as in liver disease, and dicoumarol and salicylate therapy, barium sulfate adsorbs a greater amount of nitrogenous material from plasma.

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# Adenosine Triphosphate from *Drosophila melanogaster*

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Received August 11, 1947

## INTRODUCTION

Adenosine triphosphate (ATP) from mammalian tissue has been isolated and characterized. Although pyrophosphate linkages were demonstrated early in the isolation work on ATP in some invertebrates (1), this compound has been extracted in pure form from these tissues in only one instance, in the case of the octopus (2). Since *Drosophila melanogaster* has been widely used in genetic work, and since it can be grown with ease in the laboratory in large quantity, an attempt was made to isolate and characterize its adenosine triphosphate. The present paper reports the results of such studies.

## EXPERIMENTAL

The procedure employed in the isolation was essentially that of Needham (3) with slight modification. A typical run is outlined below: 11.5 g. of ether-anaesthetized *Drosophila melanogaster* were homogenized in approximately 10 ml. of iced 10% trichloroacetic acid with a motor driven glass homogenizer, and the protein removed by centrifugation. To the orange-pigmented supernatant fluid was added an equal volume of cold 95% ethyl alcohol; the precipitated glycogen was centrifuged out. To the supernatant were added 3 ml. of 25% barium acetate and the pH adjusted to 7.0 with 30% NaOH. After 0.5 hour in the cold the barium precipitate was collected and washed twice with cold 95% alcohol. (This removed most of the orange pigment.) The barium precipitate was then suspended in water, centrifuged once more and the supernatant discarded. The barium-insoluble precipitate remaining was then treated according to the method of Needham (2) for the isolation of ATP from mammalian muscle, and 20 mg. of a grayish barium salt were obtained.

The barium salt was assayed for inorganic phosphorus, labile phosphorus (phosphorus hydrolyzed in 7 minutes at 100°C. in *N* HCl) and total phosphorus, according to the method of Fiske and SubbaRow (4). Color development curves for pentose were run according to the method of Albaum and Umbreit (5). Purine was assayed spectrophotometrically.



On the assumption that the compound isolated was barium adenosine triphosphate (M. W. 853), the purity based on organic phosphorus was 78%. The molar ratio of labile phosphorus: total phosphorus: pentose: purine was 1.90: 3.00: 1.00:1.04.

That the purine was adenine is indicated by the absorption spectrum shown in Fig. 1. The absorption maximum is at 260  $m\mu$ .

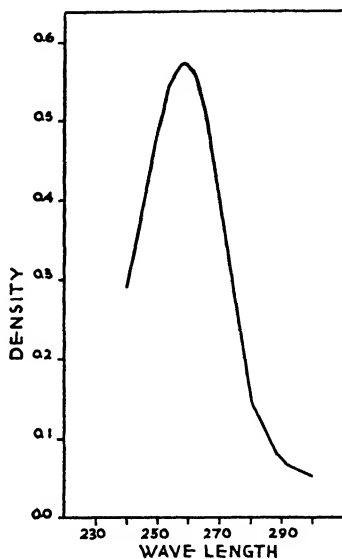


Fig. 1. Ultraviolet absorption spectrum of *Drosophila* adenosinetriphosphate. Spectrum was determined on the barium-free salt in 0.01 *M* phosphate buffer, pH 7.7 in a Beckmann spectrophotometer.

The color development curves in the orcinol-pentose reaction are shown in Fig. 2, along with curves for pure ribose-5-phosphate, arabinose-5-phosphate and xylose-5-phosphate. It is concluded that the compound contained a ribose-5-phosphate.

Proof that the labile phosphorus is of the pyrophosphate type is afforded by the experiment shown in Fig. 3, where the *Drosophila* ATP was treated with a potato pyrophosphatase prepared according to the method of Kalekar (6).

Final proof that the adenylic acid resulting from the removal of the labile phosphorus is of the muscle type (5-adenylic acid) and not the yeast type (3-adenylic acid) is shown by the experiment in Fig. 4, using a muscle deaminase prepared according to the method of Conway and

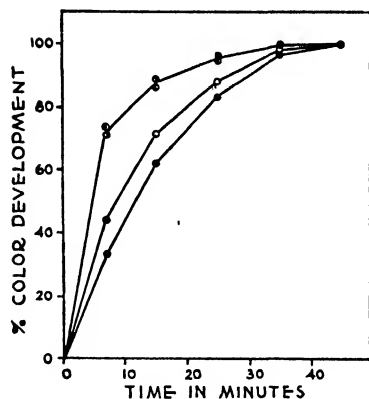


FIG. 2. Color development curves in the orcinol-pentose reaction. Each tube contained 3 ml. 12 *N* HCl, 0.123 *M* with respect to ferric ion; 0.3 ml. 10% alcoholic orcinol solution; sample and water to 6.3 ml. ● ribose-5-phosphate, ● *Drosophila* ATP, ○ xylose-5-phosphate, ⊗ arabinose-5-phosphate.

Cooke (7). It should be noted that the enzyme is without effect on yeast adenylic acid, has slight activity on the *Drosophila* ATP, but rapidly deaminates *Drosophila* ATP first treated with the potato pyrophosphatase, as well as pure muscle adenylic acid.

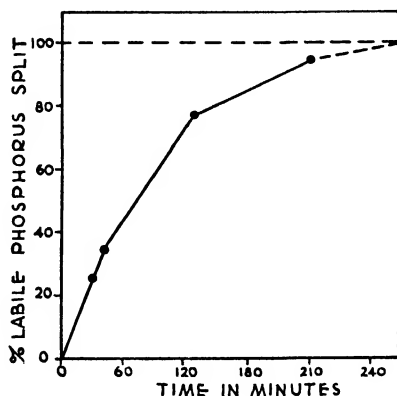


FIG. 3. Effect of potato pyrophosphatase on *Drosophila* adenosinetriphosphate. The 100% line represents 2/3 of the total organic phosphorus of the preparation. The reaction mixture in addition to enzyme and sample contained 0.1 ml. 0.1 *M* malonate buffer, pH 5.9, 0.1 ml. CaCl<sub>2</sub> solution containing 100  $\gamma$  Ca/ml., and water in a final volume of 0.8 ml.

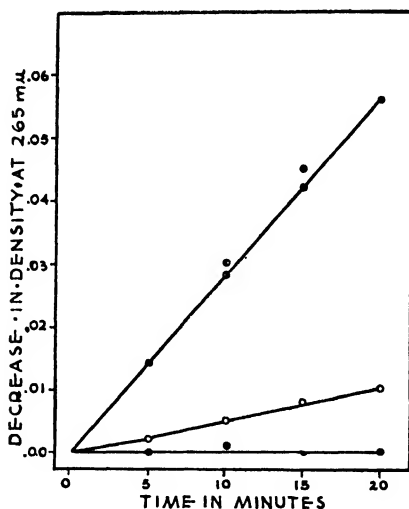


FIG. 4. Effect of muscle deaminase on *Drosophila* adenosinetriphosphate ○, *Drosophila* adenosinetriphosphate first treated with potato pyrophosphatase ○, muscle adenylic acid ●, and yeast adenylic acid ⊗. The determinations were carried out in the Beckmann spectrophotometer at 265 mμ. Each reaction mixture contained sample, 0.2 ml. of the deaminase, 0.5 ml. 0.1 M malonate buffer, pH 5.9, and water to a final volume of 5.0 ml.

Physiological activity for the *Drosophila* ATP is demonstrated by the experiment in Table I which shows that it can be used to phos-

TABLE I

*Phosphorylation of Glucose by Yeast Hexokinase in the Presence of Drosophila Adenosinetriphosphate*

Each tube contained *Drosophila* adenosinetriphosphate, 0.1 ml. 0.5 M glucose, 0.75 ml. hexokinase (4 mg./3 ml. water), 0.05 ml. 0.1 M NaHCO<sub>3</sub>, and 0.2 M MgCl<sub>2</sub>, 0.05 ml. 0.54 M NaHCO<sub>3</sub>, and water to a final volume of 2 ml. The reaction was carried out for 45 minutes at room temperature.

Initial pyrophosphate phosphorus in sample	24.2 γ
Pyrophosphate phosphorus after 45 minutes	14.2 γ
Increase in inorganic phosphorus	0.0 γ
Increase in organic phosphorus after 45 minutes (by determination)	10.0 γ
Pyrophosphate phosphorus available for reaction— $\frac{1}{2}$ of total	12.1 γ
Per cent of available phosphorus used in reaction at end of 45 minutes	83%

phorylate glucose to glucose-6-phosphate in the presence of hexokinase.<sup>1</sup>

### SUMMARY

Adenosine triphosphate isolated from *Drosophila melanogaster* appears to be identical with that obtained from mammalian muscle. This identity has been established on the basis of molar ratio of labile phosphorus: total phosphorus: pentose: purine; color development in the pentose-orcinol reaction; purine absorption spectrum; action by pyrophosphatase and muscle deaminase; and by physiological activity.

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<sup>1</sup> Obtained from Dr. M. Kunitz, Rockefeller Institute, Princeton, N. J.



## Microbiological Determination of Cytosine, Uracil and Thymine <sup>1</sup>

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Received August 27, 1947

Since Richardson's (1) discovery of the anaerobic requirements of uracil by a strain of *Staphylococcus aureus*, the naturally occurring pyrimidines have been found to be of importance in the nutrition of lactic acid bacteria (2) and a wide variety of other organisms (3). Because of the physiological importance of these compounds and the lack of quantitative methods for their determination,<sup>2</sup> it seemed worth while to investigate further the pyrimidine requirements of microorganisms leading to the development of assay procedures.

A total of 123 strains of bacteria were tested over incubation periods of 12 to 120 hours for their requirements of cytosine, uracil and thymine. The growth rates of many of the organisms were increased by the presence of one or more of these substances but, on the media employed, only 6 strains showed strict requirements. *Lactobacillus brevis* 8257,<sup>3</sup> *L. pentoaceticus* 367, and *L. fermentatus* 4006 responded equally to cytosine and uracil while *L. helveticus* 335, *L. helveticus* 6345, and *Streptococcus lactis* 7963 were specific for uracil. On testing nine of these organisms on the same media with the omission of folic acid, *S. lactis* 8022, *S. lactis* 8043, *S. faecalis* 9790, *L. helveticus* 335, and *L. helveticus* 6345 were found to be specific for thymine under these conditions. This is in agreement with Stokes (5) who reported that several strains of *streptococci* and *lactobacilli* were able to utilize thymine in place of folic acid. The activities of intact nucleic acids, nucleosides, and related compounds were not determined.

<sup>1</sup> Paper 42. For Paper 41 see Yeh, Frankl, Dunn, Parker, Hughes and György, in press. This work was aided by grants from the Nutrition Foundation and the University of California.

<sup>2</sup> A promising colorimetric procedure for cytosine and uracil has been reported by Soodak and Cerecedo (4).

<sup>3</sup> Signifies the American Type Culture Collection number for this and other organisms.

Using the standard microbiological techniques of this laboratory, synthetic basal media and assay conditions were established for the determination of uracil and thymine with *L. helveticus* 335, and for uracil plus cytosine with *L. brevis* 8257. The medium for *L. helveticus* 335 is similar to that of Dunn *et al.* (6), except for the addition of 0.001% of hypoxanthine and the omission of ammonium chloride. For *L. brevis* 8257, this medium was further modified by substituting 3% of arabinose (7) for 2% of glucose. The incubation temperature was 32°C. and the incubation times were 120 hours for *L. helveticus* 335, and 96 hours for *L. brevis* 8257. In all cases, the pyrimidines not being determined were included in the basal medium at a level of 0.001%. The acid-hydrolyzed test mixture employed contained all of the constituents in the basal medium in proportions simulating those in natural products. The concentration of the uracil, cytosine, thymine, or uracil plus cytosine in the recovery solutions was 0.5% of the dry weight of the test mixture, while that of the other pyrimidines was 1 per cent.

Recoveries of  $101 \pm 1\%$ ,  $104 \pm 4\%$ , and  $103 \pm 3\%$  for cytosine, uracil, and cytosine plus uracil, respectively, were obtained with *L. brevis* 8257 (Table I) over the range of 1  $\gamma$ –15  $\gamma$ /3 ml. tube and recoveries of  $100 \pm 6\%$  for uracil and  $98 \pm 5\%$  for thymine were found

TABLE I  
Recoveries of Cytosine and Uracil from Test  
Mixtures with *Lactobacillus brevis* 8257

Tube number	Pyrimidine present ( $\gamma$ /3 ml. tube)	Titrations <sup>a</sup> (ml. 0.0300 N NaOH/ 3 ml. tube)			Pyrimidine found ( $\gamma$ /3 ml. tube)			Pyrimidine recovery (Per cent)		
		Cyto- sine	Uracil	Cyto- sine + uracil	Cyto- sine	Uracil	Cyto- sine + uracil	Cyto- sine	Uracil	Cyto- sine + uracil
1	0	0.47	0.43	0.47	—	—	—	—	—	—
2	1.00	2.23	2.31	2.16	1.00	1.10	1.00	100	110	100
3	2.50	4.78	4.79	4.97	2.51	2.52	2.60	100	101	104
4	5.00	8.92	8.56	8.89	5.02	4.80	4.98	100	96	100
5	10.00	16.36	17.17	16.33	10.28	11.00	10.23	103	110	102
6	15.00	22.35	22.74	23.80	15.35	15.70	16.75	102	105	111
Average								101	104	103

<sup>a</sup> Averages of duplicate tubes.

with *L. helveticus* 335 over the ranges 0.3  $\gamma$ –3.0  $\gamma$  and 0.25  $\gamma$ –1.0  $\gamma$ , respectively,/3 ml. tube. Since thymine, uracil, and cytosine were recovered satisfactorily from test mixtures, it should be possible to determine these pyrimidines in natural products by adaptations of the described methods.

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# Effect of High-moisture Storage on Preservation of Carotene in Dehydrated Alfalfa Meal<sup>1</sup>

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Received August 28, 1947

## INTRODUCTION

Dehydrated alfalfa meal is used extensively as a source of vitamin A (as carotene) in the rations of animals. The carotene content of alfalfa meal decreases rapidly under normal storage conditions, however (1). Nutritionists, therefore, are greatly interested in the problem of preventing the destruction of this valuable constituent of alfalfa.

Evidence has been presented by Silker, Schrenk and King (2) and by Bielefeldt (3) which indicates that dehydrated alfalfa meal containing a high moisture content loses less carotene during storage than does meal of low moisture content. The experiments herein described were performed to study in greater detail the relationship of moisture content to the retention of carotene during storage.

## STORAGE OF MEALS AT VARIOUS MOISTURE LEVELS

Commercial dehydrated alfalfa meal was spread in a thin layer on paper in a constant-temperature room at 25°C. Pans of water placed on the floor, with a stream of air blown across them by an electric fan, produced high humidity in the room. Portions of alfalfa were removed at intervals and placed in closed containers. Moisture determinations were made on the samples thus obtained, together with a sample of the original meal. A portion of the original meal was placed in a vacuum desiccator and subjected to reduced pressure overnight by means of a vacuum pump. This resulted in a meal containing considerably less moisture than is normally encountered commercially.

After establishing the moisture and carotene content of each sample thus obtained, the meals were transferred to 4-oz. bottles. These were closed with screw caps having an inner waxed paper disc to insure a tight seal. Each bottle had approximately one inch of air space over the meal. The samples at the beginning of storage contained

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<sup>1</sup> Contribution 346, Dept. of Chemistry, Kansas State College. This work was supported by the Kansas Industrial Development Commission.

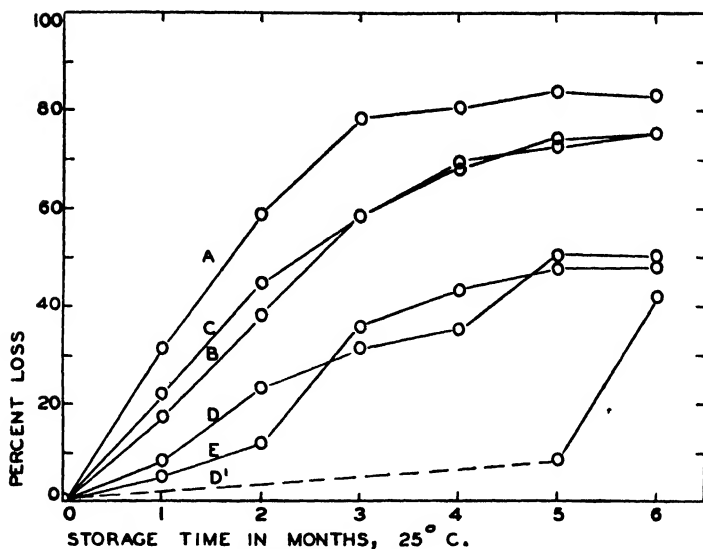


FIG. 1. Loss of carotene in alfalfa meals of varying moisture content when stored at 25°C. A, 2.7% moisture; B, 7.6%; C, 11.2%; D and D', 15.9%; E, 16.6%. Sample D' was opened for the first time after 5 months of storage.

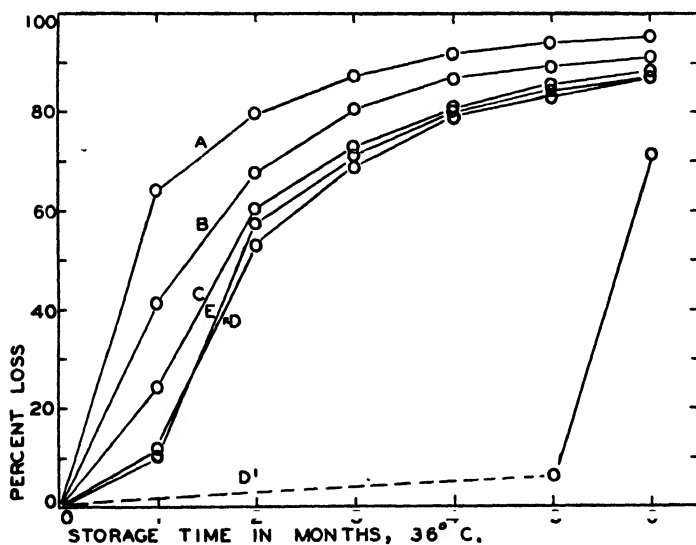


FIG. 2. Loss of carotene in alfalfa meals of varying moisture content when stored at 36°C. A, 2.7% moisture; B, 7.6%; C, 11.2%; D and D', 15.9%; E, 16.6%. Sample D' was opened for the first time after 5 months of storage.

2.7, 7.6, 11.2, 15.9, and 16.6% moisture. One bottle of each moisture level was stored at 25°C. and one each at 36°C. These were opened at monthly intervals and sampled for moisture and carotene determinations. An additional bottle of the 15.9% meal was stored at each of the two temperatures and opened for analysis for the first time after 5 months. Carotene determinations were made by the method of Silker, Schrenk and King (4).

The results of this experiment, presented in Figs. 1 and 2, show that the moisture content of the meal had a great influence on the rate of carotene destruction during storage. The lower the moisture content, the greater was the destruction of carotene. The samples which were not opened at all during the first 5 months of storage (sample D') lost very little carotene, even when stored at 36°C. Subsequent storage, after being opened at 5 months, resulted in rapid destruction of carotene. This suggests that the atmosphere in the bottle was depleted of oxygen during storage, perhaps by a fermentation process. Since the destruction of carotene in alfalfa meal has been shown to be aerobic (5), this would explain the excellent retention of carotene for 5 months in the unopened bottle, and its rapid destruction thereafter.

Sample D at the end of one month and Sample D' at the end of 5 months had lost the same amount of carotene. However, during the month following the first opening of these samples, sample D' lost carotene at a greater rate than sample D. This difference was more pronounced at the lower storage temperature.

Further evidence that fermentation had occurred was furnished by the color, odor, and pH of the samples. Measurements of pH on water extracts of the meals revealed slightly lower values for the high moisture samples. The high moisture meals became olive-green in color and had an odor reminiscent of silage, or perhaps of molded, wet hay. The meals containing 7.6% or less, moisture, retained the bright green color and characteristic odor of the original meal. It is known that organic acids, such as lactic and butyric, are produced during fermentation. The formation of such acids would account for the change in color and odor of the high moisture meals. In the presence of acid, chlorophylls are converted to pheophytins, with a resultant change in color (6).

#### EFFECT OF ACID AND ALKALI ON CAROTENE DESTRUCTION DURING STORAGE

It is seen from the first two figures that a wide variation in carotene destruction occurred after the first month in those samples which were opened to the air at monthly intervals. This variation after the first month cannot be accounted for on the basis of lack of oxygen. Since

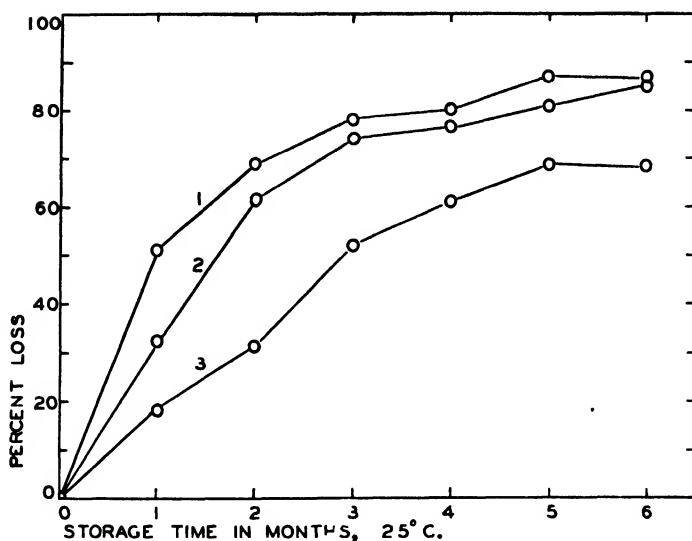


FIG. 3. Loss of carotene in alfalfa meals sprayed with water, 5% lactic acid, and 0.1 N NaOH when stored at 25°C. 1, Alkali-sprayed; 2, water-sprayed; 3, acid-sprayed.

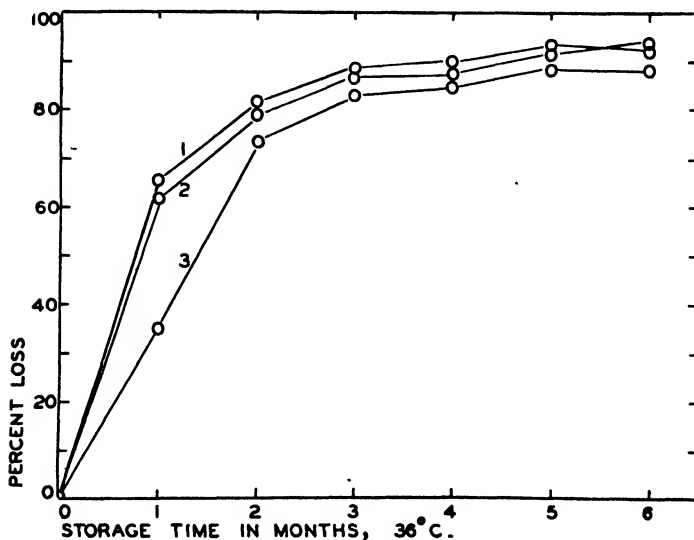


FIG. 4. Loss of carotene in alfalfa meals sprayed with water, 5% lactic acid, and 0.1 N NaOH when stored at 36°C. 1, Alkali-sprayed; 2, water-sprayed; 3, acid-sprayed.

acids apparently had been produced during fermentation, an experiment was performed to determine the effect of acid on the stability of carotene in alfalfa meal.

Lactic acid was selected because it is frequently produced during fermentations. Alfalfa meal was spread in a thin layer on paper. The meal was thoroughly moistened with a 5% solution of lactic acid by means of a small hand sprayer. Other samples were sprayed with water and with 0.1 *N* NaOH. The meals were dried in a vacuum oven overnight at 50°C. The final moisture contents were similar (about 6%), so that moisture was not a factor to be considered in the experiment. The meals were stored at 25°C. and 36°C. in 4-oz. screw cap bottles. Carotene determinations were made at monthly intervals. The results of the experiment are shown in Figs. 3 and 4.

Spraying with lactic acid reduced carotene destruction, while spraying with NaOH increased it, when compared with the control sample sprayed with water. The acid-sprayed meal became olive-green in color, while the water- and alkali-sprayed meals did not undergo a color change.

It seems probable from this experiment that the acids produced during fermentation can contribute to the retention of carotene. If the meal is not stored in an air-tight container, the effect of the acids will be the major effect. Acid production during fermentation may partially account for the results previously reported (2, 3), in which air-tight containers were not used.

## DISCUSSION

In this publication, fermentation has been advanced as the reason for the greater stability of carotene in dehydrated alfalfa meals of high moisture content. The enzymes necessary for fermentation may be of two sources: The dehydration process may not inactivate all of the enzymes which are present in alfalfa (7), or bacteria and fungi may grow on the moist meals, producing the enzymes necessary for the reactions to occur. Whatever the source may be, it is apparent from Fig. 1 that the meal containing 11.2% moisture retained carotene no better than did the original meal (7.6% moisture). Hence, moisture contents below this value do not permit sufficient fermentation to improve the stability of the carotene.

Storage of dehydrated alfalfa meal with a high moisture content may be a practical way of reducing the loss of carotene that occurs under present methods of storage. The changes in color and odor may be objectionable from the standpoint of buyer prejudice, however. No

information is available concerning the palatability and nutritive value of these meals. Additional research, therefore, will be necessary to evaluate adequately the possibilities of such storage.

#### SUMMARY

Dehydrated alfalfa meals with a high moisture content lost less carotene during storage than did meals of low moisture content. A meal containing 15.9% moisture lost 9% of its carotene when stored at 25°C. in air-tight containers and opened for the first time after 5 months. A corresponding sample opened at monthly intervals lost considerably more of its carotene (50%), but less than was lost by meals of lower moisture content under similar conditions (73–84%). Color, odor, and pH changes of the high moisture meals indicated that fermentation had occurred during storage, resulting in an oxygen-deficient atmosphere.

Evidence was presented which indicated that the acids formed during fermentation markedly increased carotene retention, even when oxygen was present.

The moisture content necessary for adequate preservation of carotene was above 11.2%. The possibility of reducing carotene destruction by storing meals with a high moisture content was discussed.

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# The Amylase of *Bacillus polymyxa*

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Received September 3, 1947

## INTRODUCTION

In connection with extensive studies on the fermentation of grain mash with *Bacillus polymyxa* to produce 2,3-butanediol (1, 2, 3, 4, 5), attention has been drawn to the active amylolytic activity of many strains of this organism. The published data on the amylase of *B. polymyxa* are not extensive. Tilden and Hudson (6) described a method for its preparation and partial purification, and showed that it converted starch to reducing substances and had an optimum pH of 6.8 and temperature of 40°C. Kneen and Beckord (7) included this enzyme in an extensive study of bacterial amylases and concluded that its action on starch was similar to that of the malt amylases. The present paper presents the results of a study of the action of this amylase on starch as influenced by the substrate concentration, the addition of maltose, and the temperature.

## MATERIALS AND METHODS

The organism used throughout this study was a strain of *B. polymyxa* obtained from the National Research Council, Ottawa (N.R.C. strain C38 (2)). Preliminary investigations of the conditions most favorable for amylase production by this organism led to the use of a medium containing 4% soluble starch, 0.5% yeast extract (Difco), small amounts of added salts ( $\text{KH}_2\text{PO}_4$ , 0.055%;  $\text{Na}_2\text{HPO}_4$ , 0.014%;  $\text{MgSO}_4$ , 0.025%) and 0.5% suspended calcium carbonate. The cultures were maintained at 30°C. during the fermentation period and for several days thereafter. Under these conditions the amylolytic activity of the medium reached a maximum in about 13 days.

Estimates of the amylase activity were made by determining the time required to hydrolyze 1 mg. of starch to the achroodextric stage, or, more accurately, by determining the reducing power of the hydrolyzate by means of the sugar reagent of Underkoffler *et al.* (8).

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Except where otherwise stated in the text, all determinations of amylase activity were made at pH 6.8 in a water bath maintained at 40°C. Ten ml. of *M*/15 phosphate buffer solution, the required volume of a 1% solution of starch (Merck & Co.) and sufficient water to make 20 ml., were placed in a 150-ml. boiling tube and allowed to equilibrate to the temperature of the bath. Five ml. of the preparation of the enzyme, previously warmed to the same temperature, were then added. Five ml. samples were withdrawn by pipette, as required, for analysis. Suitable control mixtures containing heat-inactivated enzyme were incubated concurrently.

### *Preparation of the Enzyme*

The amylase produced by this organism is relatively stable in solution but no attempt was made to prepare a dry or crystalline product. For the preparation of the active solution used throughout these studies the fermented medium was first clarified by centrifugation, followed by filtration, repeated if necessary, through "Hyflo supercel" (Johns Manville Co., Toronto). The resultant filtrate was concentrated to about one-half its original volume by pervaporation, filtered again, chilled in the refrigerator at 5°C. and mixed with an equal volume of cold acetone. The precipitate was removed as rapidly as possible by centrifugation in previously chilled containers and was resuspended in cold water to give a cloudy preparation which was again clarified by filtration through "Hyflo supercel." The resultant solution was stable for several weeks under toluene at 5°C.

## EXPERIMENTAL

### *Maltase Activity*

The medium from the culture of this organism showed some maltase activity, which it was desirable to eliminate before undertaking a study of the properties of the amylase. Considerable preliminary work was, therefore, done in an attempt to separate these two enzymes; most of the adsorbents employed tended to adsorb the amylase but no suitable method of elution was found. It was noted, however, that the use of "Hyflo supercel" in the filtration of solutions containing the amylase reduced the maltase activity. Apparently this material selectively removes the maltase, and its repeated use, as suggested above, yielded an active amylolytic solution which was almost entirely free from maltase. The maltase activity of the preparation was tested by allowing the solution of the enzyme to act upon pure maltose solution under the same conditions as outlined for the hydrolysis of starch. Data for two different preparations of the enzyme are given in Table I.

TABLE I  
*Activity of Purified Amylase Solution on Maltose*

Time Min.	Reducing power, ml. 0.5 <i>N</i> Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub>	
	Exp. I <sup>a</sup>	Exp. II <sup>b</sup>
0	3.56	2.86
15	3.58	2.86
30	3.54	2.88

<sup>a</sup> 0.16% maltose, pH 6.8, 40°C., 5 ml. amylase solution in a total volume of 20 ml.

<sup>b</sup> 0.13% maltose, pH 6.8, 40°C., 5 ml. amylase solution in a total volume of 25 ml.

*Effect of pH on the Activity of the Amylase*

A phosphate buffer at pH 6.8, the optimum value suggested by Tilden and Hudson (6), was used during most of these studies, but the pH of the medium could be varied from 6.2 to 7.5 without markedly affecting the rate of hydrolysis. Beyond these limits the activity of the enzyme declined only gradually and considerable hydrolysis occurred, even at pH 4.3. Acetate and veronal buffers were also employed, but the type of buffer solution appeared to be without effect on the activity of the enzyme.

*Effect of Substrate Concentration*

To determine the effect of the concentration of starch on the rate of hydrolysis, quadruplicate experiments were conducted with concentrations varying from 0.02 to 0.16%. The procedure outlined above was followed and four samples were withdrawn at 1-min. intervals for determining the reducing power. The data thus obtained indicated that, except for the lower starch concentrations (below 0.06%), where hydrolysis approached the equilibrium level within 3 minutes, the rate of hydrolysis was practically constant for the first 2-3 mins. The reducing power of the 1-min. sample was, therefore, assumed to be a true index of the initial velocity of the enzyme action.

When the results presented in Table II were plotted according to the method of

TABLE II  
*Initial Velocity of Hydrolysis in Relation to Concentration of Substrate*  
Single determinations

Starch conc.	Mg. maltose,* 1-min. sample					Calculated values
	Exp. I	Exp. II	Exp. III	Exp. IV	Av.	
<i>Per cent</i>						
.02	0.68	0.61	0.71	0.66	0.67	0.66
.03	0.90	0.80	0.85	0.80	0.84	0.82
.04	0.94	0.91	1.01	0.94	0.95	0.94
.06	1.08	1.07	1.15	1.04	1.09	1.09
.08	1.21	1.21	1.25	1.15	1.21	1.19
.10	1.34	1.25	1.27	1.20	1.27	1.26
.16	1.48	1.41	1.46	1.32	1.42	1.38

\* Increase in reducing power calculated as maltose.

Lineweaver and Burk (9) a straight line graph was obtained. After calculation of the slope of this line the apparent dissociation constant ( $K_m$ ) of the enzyme-substrate complex (the starch concentration being expressed as per cent) was found to be 0.030.

*Effect of Added Maltose*

Since maltose is probably the principal sugar formed during the hydrolysis of starch by the purified enzyme, the presence of added maltose might be expected to retard the hydrolysis. Experiments were therefore carried out with mixtures containing 0, 0.08, 0.16, and 0.24% added maltose and the data indicated decreases of 12,

20, and 29%, respectively, in the rate of the amylolytic reaction. Over the range studied, the decrease in rate was approximately proportional to the amount of maltose added, and it was, therefore, possible to calculate the apparent affinity of the enzyme for maltose. Assuming a  $K_m$  value of 0.030 for starch, and applying the formula of Michaelis and Menten (10), values were obtained for the apparent dissociation constant of the enzyme-maltose complex of 0.077, 0.083 and 0.075, with maltose concentrations of 0.08, 0.16 and 0.24%, respectively. On the basis of these determinations the affinity of the enzyme for maltose appears to be about 12.8 (1/0.078) as compared with a value of 33.3 (1/0.030) for the affinity of the enzyme for starch.

### *Effect of Temperature*

The rate of hydrolysis, as determined by the reducing power of the 1-min. sample, was determined over a temperature range of 5°–60°C. An apparent "optimum" temperature was found at about 45°C. Inactivation of the enzyme was extremely rapid above 55°C.

When reciprocals of the absolute temperature were plotted against logarithms of the initial velocities of hydrolysis ( $1/T$  against  $\log V$ ) a straight line relation was shown over the range of 10°–40°C. It therefore appeared possible to determine the apparent "energy of activation" of the reaction over this range. To increase the rate of hydrolysis at the lower temperatures a more concentrated solution of the enzyme was used. Two concentrations of starch were used, 0.40% to saturate the enzyme and 0.20% to leave it slightly unsaturated. The values obtained for both concentrations of substrate at 13 different temperatures are presented in Table III. The regression coefficient

TABLE III

*Effect of Temperature on the Rate of Hydrolysis of Two Concentrations of Starch*  
Duplicate determinations as shown

Temperature °C.	Mg. maltose,* 1-min. samples			
	0.40% starch		0.20% starch	
10.0	0.87	0.85	0.82	0.82
12.5	0.94	0.94	0.87	0.82
15.0	0.99	1.01	0.96	0.94
17.5	0.99	1.01	0.94	0.89
20.0	1.15	1.11	1.01	1.01
22.5	1.13	1.18	1.13	1.11
25.0	1.39	1.37	1.32	1.32
27.5	1.41	1.39	1.27	1.25
30.0	1.67	1.65	1.48	1.55
32.5	2.05	2.02	1.76	1.74
35.0	2.05	2.05	1.76	1.81
37.5	2.38	2.40	2.16	2.09
40.0	2.42	2.35	2.23	2.19

\* Increase in reducing power calculated as maltose.

cients for the relation between  $1/T \times 10^4$  and  $\log V$ , calculated from these data, were  $-1522 \pm 296$  and  $-1380 \pm 297$  for the high and low concentrations of starch, respectively. The difference between these values was found not to be statistically significant and the average value,  $-1451$ , therefore represents the slope of the line for the combined data. The apparent "energy of activation" (calculated by the method given by Sizer, 11) for the reaction of this bacterial amylase in the hydrolysis of starch under these conditions is, therefore, approximately 6750 cal.

### *Degree of Hydrolysis Induced by the Enzyme*

The relatively great activity of this preparation of the *B. polymyxa* amylase, together with its freedom from maltase, should make it possible to determine the extent of hydrolysis exactly, but only preliminary studies have been made. With limiting substrate concentrations, 0.10% or less, the rate of hydrolysis declined rapidly after the first 3 or 4 minutes and the total reducing power appeared to approach a maximum value asymptotically. Under the conditions employed this maximum value was about 80% of the theoretical value representing complete conversion of the starch to maltose.

### DISCUSSION

The amylase of *B. polymyxa* appears to differ from the better known plant and bacterial amylases in several characteristics. The pH optimum has been reported as 6.8 (6) and this value is well within the wide optimum range found in the present work. It is thus well above the optimum for malt amylase (4.5–4.8 (12)), for the amylase of *B. macerans* (5.0–6.0 (13)), and for that of *Clostridium acetobutylicum* (4.8 (14), 4.8–5.0 (15)). The amylase of *Aspergillus oryzae* also has a more acid pH optimum (3.0–4.0 (16)) than that of *B. polymyxa*.

The value of 0.030 for the dissociation constant of the enzyme-substrate complex, as determined in this study, indicates a considerably higher affinity of the amylase for starch than has been reported for malt amylase ( $K_m$  0.077 (17)). Hockenull and Hebert (14) have reported a value of 0.208 for the amylase of *Cl. acetobutylicum*, but this value was calculated from the amount of hydrolysis occurring during a 30-min. period rather than from the initial velocities. Johnson and Wynne (15) had previously reported a value of 0.050 for the *Clostridium* amylase.

The apparent "energy of activation" for the hydrolysis of starch by the amylase of *B. polymyxa* (6750 cal.) is approximately the same as that reported for the enzyme of *Cl. acetobutylicum* at the low concentration of substrate (15). An "E" value of 12,300 cal. has been reported for malt amylase (11).

Kneen and Beckord (7) state that the production of fermentable sugar by the enzyme of *B. polymyxa* is similar to that obtained with malt amylase. However, for their purposes a pure amylase was not required, and they were presumably working with a mixture of enzymes, including maltase. Hockenhull and Hebert (14) conclude that the amylase of *Cl. acetobutylicum* is capable of producing the theoretically maximum quantity of maltose from starch but state that their preparation of the enzyme was contaminated with maltase. In the present work the amylase was obtained free from maltase and the reducing power of the hydrolysis mixture should, therefore, be a more reliable index of the extent of the hydrolysis of the starch. Since this reducing power did not exceed 80% of the theoretical, it is probable that a portion of the starch is not completely converted to maltose by this enzyme.

#### ACKNOWLEDGMENTS

The author wishes to thank Dr. A. M. Wynne of the Department of Biochemistry, University of Toronto, for his encouragement and instruction, and his associates in the Division of Applied Biology, National Research Council, Ottawa, for their interest in the project and assistance in the preparation of the data for publication.

#### SUMMARY

1. A stable, partially purified amylase preparation, free from maltase activity, has been obtained from cultures of *B. polymyxa*. This amylase induced a rapid hydrolysis of soluble starch, but apparently did not carry the reaction to completion.

2. The amylase showed an optimum pH range between 6.2 and 7.5, a  $K_m$  of 0.030 (starch concentration expressed as per cent), is partially inhibited by the addition of maltose to the hydrolysis mixture, and has an apparent "energy of activation" of 6750 cal. for the hydrolytic reaction.

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# Sulfur Amino Acid Nutrition of Some Lactic Acid-Producing Bacteria<sup>1</sup>

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Received September 5, 1947

## INTRODUCTION

It is the purpose of the present paper to present data on the methionine, cystine, and homocystine requirements and interrelationships of the organisms *Lactobacillus arabinosus* 17-5,<sup>3</sup> *Lactobacillus fermenti* 36, *Lactobacillus casei*, *Streptococcus faecalis* R, and *Leuconostoc mesenteroides* P-60.

Several papers on the determination of methionine (1, 2, 3, 4) have appeared recently. Considerable data have been presented on the requirements of different microorganisms for methionine and the inability of related products to replace methionine for these organisms

<sup>1</sup> Published as Scientific Paper No. 725, College of Agriculture and Agriculture Experiment Stations, Institute of Agricultural Sciences, State College of Washington, Pullman, Washington. The author wishes to thank Dr. B. L. Hutchings of Lederle Laboratories, Pearl River, N. Y., for a gift of synthetic *L. casei* factor, Dr. D. A. Greenwood of the Utah State Agricultural College, and Dr. M. S. Dunn of the University of California at Los Angeles, for gifts of proline which made this investigation possible. This paper was presented at the Northwestern Regional Meeting of the American Chemical Society in Moscow, Idaho, 1947.

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<sup>3</sup> The bacteriological cultures were obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D. C. The cultures and the American Type Culture Collection numbers used were:

<i>Lactobacillus arabinosus</i> 17-5	8014
<i>Lactobacillus casei</i>	7469
<i>Lactobacillus fermenti</i> 36	9338
<i>Streptococcus lactis</i> R ( <i>faecalis</i> R)	8043
<i>Leuconostoc mesenteroides</i> P-60	8042



(1, 2, 3, 5, 6, 7, 8). Little has been reported on the cystine or homocystine requirements of the lactic acid-producing bacteria.

### EXPERIMENTAL

The method of procedure was similar to that generally used. The medium of Schweigert *et al.* (9) was used for *L. arabinosus*, that of Baumgarten *et al.* (10) for *S. faecalis*, that of Dunn *et al.* (11) for *L. fermenti*, that of Riesen *et al.* (2) for *L. mesenteroides*, and the medium of Roberts and Snell (12) was modified for *L. casei* by replacing the casein hydrolyzate with an amino acid mixture. Growth was determined in all cases by titration of the lactic acid produced. None of the basal media used contained any of the sulfur amino acids or choline. Tubes were supplemented singly and in combination with cystine, methionine, and homocystine at levels of 30, 60, 90, and 120  $\gamma$  and 1 mg. per tube. Choline chloride was added at a 1 mg. per tube level to half of the tubes to which homocystine was added. The final volume of each tube was 10 ml. Commercial DL-methionine and L-cystine were used. DL-Homocystine was prepared from DL-methionine by the procedure of du Vigneaud, Loring and Craft (13).

The results of the study are summarized in Table I. Only data for the 60  $\gamma$  and 1 mg. levels are given, since these are representative.

TABLE I

*Growth Response of Some Lactic Acid-Producing Bacteria to Cystine, Methionine, Homocystine, and Combinations of These*  
(Ml. of 0.1 N Sodium Hydroxide for Titration)

Supplementary amino acids	<i>L. arabinosus</i>		<i>S. faecalis</i>		<i>L. fermenti</i>		<i>L. casei</i>		<i>L. mesenteroides</i>	
Level in medium	60 $\gamma$	1 mg.	60 $\gamma$	1 mg.	60 $\gamma$	1 mg.	60 $\gamma$	1 mg.	60 $\gamma$	1 mg.
<i>Methionine-free medium</i>										
None	3.7		2.1		0.3		2.3		0.5	
Methionine	9.7	10.7	7.6	7.9	4.2	12.9	8.7	8.7	5.0	4.9
Homocystine	3.8	8.4	1.9	2.3	0.3	0.3	2.9	4.5	0.4	0.4
Choline (1 mg.) + homocystine	4.3	8.5	2.1	2.3	0.4	0.4	6.5	8.5	0.4	0.4
Choline (1 mg.) + homocystine + methionine		11.3		8.7		1.2		9.8		4.8
<i>Cystine-free medium</i>										
None	0.2		5.2		11.8		1.9		0.6	
Cystine	4.6	10.7	5.9	7.9	13.8	12.9	3.2	8.7	4.4	4.9
Homocystine	0.0	0.6	5.2	6.2	10.5	2.0	1.7	2.9	0.6	1.4
Choline (1 mg.) + cystine + homocystine		11.3		8.7		1.2		9.8		4.8

## RESULTS AND DISCUSSION

*L. arabinosus* 17-5 required both methionine and cystine for optimum growth. Neither homocystine nor homocystine plus choline could replace methionine or cystine. The need of *L. arabinosus* for cystine is contrary to observations of Riesen (14) and of Dunn *et al.* (5) that *L. arabinosus* required methionine but not cystine, but is in agreement with observations of Barton-Wright (15) who used *L. arabinosus* for cystine assays.

*S. faecalis* R required methionine but not cystine or homocystine for growth. Both cystine and homocystine slightly stimulated acid production when added at a level of 1 mg. to tubes containing methionine.

*L. fermenti* 36 required methionine but not cystine for growth. Growth was inhibited by the addition of 1 mg. of homocystine to each tube of complete media (media containing 1 mg. per tube of methionine). This inhibition by homocystine was observed only with *L. fermenti* of the organisms studied. The methionine metabolism of *L. fermenti* is unique, also, in that it can utilize D-methionine as well as L-methionine according to Dunn and coworkers (1). Ethionine (16) and methoxenine (17), compounds structurally related to methionine, inhibit growth of *E. coli* and *Staph. aureus*. Harris and Kohn (16) attribute this inhibition to metabolic competition with methionine.

*L. casei* required both methionine and cystine for growth in agreement with data of Hutchings and Peterson (7) but in disagreement with data of Dunn *et al.* (5). Homocystine plus choline stimulated growth at the 1 mg. per tube level when the media contained cystine, methionine, both, and neither. Choline chloride plus 60  $\gamma$  of homocystine gave a growth response equal to that given by 30  $\gamma$  of methionine. Homocystine plus choline can replace methionine for certain monocellular animal organisms (18), but homocystine plus choline will not replace methionine for a strain of *E. coli* requiring methionine for growth (19).

*L. mesenteroides* P-60 required both methionine and cystine for growth.

## SUMMARY

*Lactobacillus arabinosus* 17-5, *Streptococcus faecalis* R, *Lactobacillus fermenti* 36, *Lactobacillus casei*, and *Leuconostoc mesenteroides* P-60 all required methionine for normal growth. Homocystine in the absence of

(1, 2, 3, 5, 6, 7, 8). Little has been reported on the cystine or homocystine requirements of the lactic acid-producing bacteria.

### EXPERIMENTAL

The method of procedure was similar to that generally used. The medium of Schweigert *et al.* (9) was used for *L. arabinosus*, that of Baumgarten *et al.* (10) for *S. faecalis*, that of Dunn *et al.* (11) for *L. fermenti*, that of Riesen *et al.* (2) for *L. mesenteroides*, and the medium of Roberts and Snell (12) was modified for *L. casei* by replacing the casein hydrolyzate with an amino acid mixture. Growth was determined in all cases by titration of the lactic acid produced. None of the basal media used contained any of the sulfur amino acids or choline. Tubes were supplemented singly and in combination with cystine, methionine, and homocystine at levels of 30, 60, 90, and 120  $\gamma$  and 1 mg. per tube. Choline chloride was added at a 1 mg. per tube level to half of the tubes to which homocystine was added. The final volume of each tube was 10 ml. Commercial DL-methionine and L-cystine were used. DL-Homocystine was prepared from DL-methionine by the procedure of du Vigneaud, Loring and Craft (13).

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(Ml. of 0.1 N Sodium Hydroxide for Titration)

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Level in medium	60 $\gamma$	1 mg.	60 $\gamma$	1 mg.	60 $\gamma$	1 mg.	60 $\gamma$	1 mg.	60 $\gamma$	1 mg.
<i>Methionine-free medium</i>										
None	3.7		2.1		0.3		2.3		0.5	
Methionine	9.7	10.7	7.6	7.9	4.2	12.9	8.7	8.7	5.0	4.9
Homocystine	3.8	8.4	1.9	2.3	0.3	0.3	2.9	4.5	0.4	0.4
Choline (1 mg.) + homocystine	4.3	8.5	2.1	2.3	0.4	0.4	6.5	8.5	0.4	0.4
Choline (1 mg.) + homocystine + methionine		11.3		8.7		1.2		9.8		4.8
<i>Cystine-free medium</i>										
None	0.2		5.2		11.8		1.9		0.6	
Cystine	4.6	10.7	5.9	7.9	13.8	12.9	3.2	8.7	4.4	4.9
Homocystine	0.0	0.6	5.2	6.2	10.5	2.0	1.7	2.9	0.6	1.4
Choline (1 mg.) + cystine + homocystine		11.3		8.7		1.2		9.8		4.8

## RESULTS AND DISCUSSION

*L. arabinosus* 17-5 required both methionine and cystine for optimum growth. Neither homocystine nor homocystine plus choline could replace methionine or cystine. The need of *L. arabinosus* for cystine is contrary to observations of Riesen (14) and of Dunn *et al.* (5) that *L. arabinosus* required methionine but not cystine, but is in agreement with observations of Barton-Wright (15) who used *L. arabinosus* for cystine assays.

*S. faecalis* R required methionine but not cystine or homocystine for growth. Both cystine and homocystine slightly stimulated acid production when added at a level of 1 mg. to tubes containing methionine.

*L. fermenti* 36 required methionine but not cystine for growth. Growth was inhibited by the addition of 1 mg. of homocystine to each tube of complete media (media containing 1 mg. per tube of methionine). This inhibition by homocystine was observed only with *L. fermenti* of the organisms studied. The methionine metabolism of *L. fermenti* is unique, also, in that it can utilize D-methionine as well as L-methionine according to Dunn and coworkers (1). Ethionine (16) and methoxenine (17), compounds structurally related to methionine, inhibit growth of *E. coli* and *Staph. aureus*. Harris and Kohn (16) attribute this inhibition to metabolic competition with methionine.

*L. casei* required both methionine and cystine for growth in agreement with data of Hutchings and Peterson (7) but in disagreement with data of Dunn *et al.* (5). Homocystine plus choline stimulated growth at the 1 mg. per tube level when the media contained cystine, methionine, both, and neither. Choline chloride plus 60  $\gamma$  of homocystine gave a growth response equal to that given by 30  $\gamma$  of methionine. Homocystine plus choline can replace methionine for certain monocellular animal organisms (18), but homocystine plus choline will not replace methionine for a strain of *E. coli* requiring methionine for growth (19).

*L. mesenteroides* P-60 required both methionine and cystine for growth.

## SUMMARY

*Lactobacillus arabinosus* 17-5, *Streptococcus faecalis* R, *Lactobacillus fermenti* 36, *Lactobacillus casei*, and *Leuconostoc mesenteroides* P-60 all required methionine for normal growth. Homocystine in the absence of

methionine stimulated growth of *L. arabinosus* and *L. casei* but not of *S. faecalis*, *L. fermenti*, and *L. mesenteroides*. Homocystine plus choline replaced methionine for growth of *L. casei* but more homocystine than methionine was required for a given growth response.

Cystine was required by *L. arabinosus*, *L. casei*, and *L. mesenteroides* for growth. It was not required by *L. fermenti* or *S. faecalis*, though it slightly stimulated growth of *L. fermenti* on a complete medium. Homocystine inhibited growth of *L. fermenti* when added to a complete medium.

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# Ultrafilterable and Non-Ultrafilterable Calcium in Normal, Hyperplastic Epidermis and Squamous Cell Carcinoma

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Received September 8, 1947

## INTRODUCTION

Suntzeff and Carruthers (1, 2) and Suntzeff, Carruthers and Cowdry (3) have established that the calcium content of mouse epidermis drops to about 50% of normal within 10 days after painting with methylcholanthrene. Further, they have shown that a transplantable squamous cell carcinoma contains approximately 50% less calcium than does the hyperplastic epidermis. Consequently, a program has been initiated to determine the nature of the decrease in calcium induced by methylcholanthrene.

The present study was designed to determine whether or not the decrease in calcium in hyperplastic and carcinomatous epidermis is accompanied by a shift in the ultrafilterable and non-ultrafilterable calcium equilibrium. Avascular epidermis is particularly favorable material for such analysis since there is no contamination by blood and its calcium-binding constituents.

## EXPERIMENTAL

Approximately 3-month old male Swiss mice were used in all of the direct epidermal determinations. Groups of 20 mice were used for each control analysis and groups of 40 for the experimental analyses. This doubling of the number of mice in the experimental groups was necessitated to compensate for the reduced amount of calcium in the hyperplastic epidermis. The experimental mice were each given 3 paintings with 0.6% methylcholanthrene in benzene and the animals were sacrificed on the twentieth day after the initial painting. Skin on the back was rapidly removed and frozen with dry ice. To remove the epidermis, the frozen skin was allowed to thaw slightly and the epidermis was scraped free with a scalpel. Separation of epidermis from dermis is

effected fairly readily at a temperature slightly below 0°C. Histological preparations of the remaining skin were used to check on the degree of separation of epidermis from dermis.

Material for tumor analysis was obtained by subcutaneous implantation in mice 6-8 weeks old of a transplantable squamous cell carcinoma which is being maintained at the Barnard Free Skin and Cancer Hospital. The tumors, when 7-8 mm. in diameter, were removed from the hosts and frozen in dry ice until used in analyses. These small tumors were free from necrotic areas.

The technique of separation of filterable from non-filterable calcium by ultrafiltration was essentially that of Updegraff, Greenberg and Clark (4), Greenberg and Gunther (5), and Mazia (6). The collodion sacs were prepared in a manner similar to that described by Mazia, except that the preparations were made in a cold room maintained at 9°C. The primary deviation from the ultrafiltration technique developed by the aforementioned workers was the use of a cold room maintained at 0.5-2.0°C. This procedure was established because early pilot experiments indicated that the amount of filterable calcium obtainable from epidermis by ultrafiltration increased sharply at temperatures near or above 5°C.

Frozen epidermis was thoroughly macerated with a mortar and pestle or with a tissue blending device and was then transferred to a collodion sac and suspended in 10 cc. of 0.85% NaCl. The ultrafiltration was continued for 24 hours at a constant negative pressure of 150 mm. Hg.

The calcium content of the ultrafilterable and non-ultrafilterable fractions was determined by the method of Lindner and Kirk (11). The calcium levels were referred to the wet weight of tissue. Results were calculated in terms of  $\gamma$  of calcium/100 mg. fresh weight of tissue. It was thus possible to standardize our analyses with the total calcium levels obtained by Suntzeff and Carruthers (1, 2) for normal, hyperplastic epidermis, and squamous cell carcinoma.

The results obtained in our experiments are itemized in Table I. The first observation of significance is that total calcium obtained by adding the ultrafilterable and non-ultrafilterable calcium fractions closely parallels the total calcium values obtained by Suntzeff and Carruthers, and that the decreases in total calcium in hyperplastic epidermis and squamous cell carcinoma are also quantitatively reproduced.

Normal epidermis contains 30.8  $\gamma$  of ultrafilterable calcium/100 mg. of wet weight epidermis which is 38.4% of the total calcium. The non-ultrafilterable calcium fraction is 49.4  $\gamma$ , or 61.6% of the total. The hyperplastic epidermis shows a drop of approximately 60% in total calcium which is reflected in both calcium fractions. Thus, the ultrafilterable calcium of hyperplastic epidermis, while reduced to 12.0  $\gamma$ , constitutes 42% of the total, and the non-ultrafilterable calcium fraction reduced to 16.7  $\gamma$  is 58% of total calcium. The per cent differ-

TABLE I

*Ultrafilterable and Non-Ultrafilterable Calcium in Normal Epidermis, Hyperplastic Epidermis, and Squamous Cell Carcinoma*

Tissue	Experiment number	A Ultrafilterable calcium, $\gamma$ /100 mg. tissue	B Non-ultrafilterable calcium, $\gamma$ /100 mg. tissue	C Ratio A:B
Normal epidermis	1	33.8	50.1	
	2	32.5	52.1	
	3	20.1	36.1	
	4	36.8	59.3	
Mean		30.8	49.4	1.6
Hyperplastic epidermis	5	10.2	15.5	
	6	14.3	17.4	
	7	8.5	14.9	
	8	15.1	19.0	
Mean		12.0	16.7	1.4
Squamous cell carcinoma	9	1.6	3.5	
	10	1.2	2.7	
	11	1.5	2.1	
	12	1.9	—	
Mean		1.5	3.7	2.4

ences between normal and hyperplastic ultrafilterable and non-ultrafilterable fractions are not considered to be significant.

The transplantable squamous cell carcinoma, in contrast to normal and hyperplastic epidermis, reveals a significant alteration in the ratio of ultrafilterable to non-ultrafilterable calcium. Ultrafilterable calcium, in this instance, is 1.5  $\gamma$ , or 29% of the total calcium, while the non-ultrafilterable fraction contains 3.7  $\gamma$  which is 71% of total calcium.



## DISCUSSION

The ultrafiltration method, as employed in this study, is capable of yielding consistent results, although the sources of error in ultrafiltration, as discussed by Mazia (6), apply, and the values for ultrafilterable calcium are relative and probably maximal. It seems clear that, under the conditions used, ultrafilterable calcium is sharply reduced in squamous cell carcinoma but not in methylcholanthrene-induced hyperplasia. From this reduction, both absolute and relative with respect to total calcium, it may be concluded that the base-binding capacity of the organic tissue fraction which binds calcium is altered in the tumor studied.

Calcium, presumably bound to a protein, is held to be an integral part of the cell periphery or cortex (Heilbrunn (7) and Mazia (8)). If it is accepted that the currently observed alteration in calcium-binding capacity is localized in the cell periphery, it becomes possible to interpret the observations of Coman (10). In proposing a mechanism of invasiveness of cancer, Coman suggested that decreased adhesiveness of cancer cells, resulting from local calcium deficiency, facilitates the separation of these cells from one another. This view was supported by his observation (9) that attached pairs of cancer cells are more readily separated by micromanipulation than are normal cells, and that normal cells in calcium-free medium are more readily separated than cells in balanced salt solution.

## SUMMARY

Ultrafiltration studies on normal and hyperplastic epidermis, and squamous cell carcinoma have been conducted. Ultrafilterable calcium of normal epidermis is 38% of total calcium. In methylcholanthrene-induced hyperplasia, the ultrafilterable calcium level is unaltered, but in squamous cell carcinoma it is reduced to 29%.

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# Comparative Study of Pteroylglutamic Acid and Its Hexaglutamyl Conjugate in the Correction of Sulfasuxidine-Induced Leucopenia in the Rat. Effect of Conjugase Inhibitor \*

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Received September 10, 1947

## INTRODUCTION

The experiments of Daft and Sebrell (1) established pteroylglutamic acid (PGA) as an essential hematopoietic agent for the rat. This animal, receiving a purified diet supplemented with a sulfonamide drug, develops a blood dyscrasia characterized by an eventual reduction in all the formed elements of the blood—white blood cells of all types, red blood cells, and platelets (2, 3, 4, 5). Administration of PGA, either orally or subcutaneously, corrects in every respect this pancytopenic state. Changes in blood morphology of rats apparently deficient in PGA parallel those observed in PGA deficient monkeys (6) and chicks (7).

Mallory (8), Stewart (9) and coworkers have obtained hematologic responses in the sulfa-treated PGA-deficient rat following the administration of yeast extracts which showed no PGA activity microbiologically except after treatment with PGA conjugase concentrates. It was suggested that the PGA conjugate was responsible for the blood changes observed in these animals.

Bird and colleagues (10) have shown that there was a substance present in yeast extracts which inhibited PGA hexaglutamyl conjugate cleavage by hog kidney conjugase. It appeared possible that this conjugase inhibitor could influence the hematologic response to PGA hexaglutamyl conjugate in the sulfa-treated PGA-deficient rat.

The purpose of the experiments reported here was to confirm the hematopoietic action of PGA conjugate using crystalline pteroyl-hexaglutamylglutamic acid, and further, to compare quantitatively the effect of this compound as well as a conjugate concentrate containing conjugase inhibitor with crystalline PGA.

\* Presented in part at the American Chemical Society Meeting, September, 1946.

## EXPERIMENTAL PROCEDURE

Weanling rats were maintained on a diet of the following percentage composition: casein purified, 18; cerelose, 66; salt mixture (Jones and Foster), 4; cotton seed oil, 6; corn oil, 2; sulfasuxidine, 2; nicotinic acid, 0.18; inositol, 0.18; thiamine, 0.014; riboflavin, 0.014; pyridoxine, 0.014; pantothenic acid, 0.18; and choline, 1.08.

Total leucocyte and granulocyte counts were made at weekly intervals. When the granulocyte count fell to 250 cells or less/mm.<sup>3</sup>, the animal was treated with either PGA or its hexaglutamyl conjugate. The effectiveness of the treatment material was measured on the basis of a test proposed by Daft (1): the minimum amount necessary to cause an increase in cell counts four days after therapy to the levels of 7,500 total leucocytes and 2,000 granulocytes/mm.<sup>3</sup>, values which represent the average of a large number of determinations in normal rats from our colony.

The urinary excretion of PGA, preceding and following the administration of the treatment materials, was also measured using a microbiological assay procedure (11). The urine was collected on filter paper and removed by repeated washings.

Crystalline PGA hexaglutamyl conjugate was obtained through the courtesy of Dr. J. J. Pfiffner of these Laboratories. The concentrate of PGA hexaglutamyl conjugate used was a Norit eluate of yeast and contained conjugase inhibitor. To split the conjugate present in this concentrate required 36 times as many PGA conjugase enzyme units as were necessary to free an equivalent amount of vitamin from crystalline hexaglutamyl conjugate.

## RESULTS AND DISCUSSION

The results of treatment with graded amounts of PGA, crystalline PGA hexaglutamyl conjugate, and concentrates of the conjugate containing conjugase inhibitor on granulocyte and total leucocyte blood levels are shown in Table I. Oral and parenteral administration of PGA and its conjugate are also compared. The values reported are the average numbers of cells, with the range of cell numbers in parentheses, obtained four days after a single administration of treatment material.

Twenty  $\gamma$  PGA given orally does not enable the production of granulocytes or total leucocytes to increase to standard levels of 2,000 granulocytes and 7,500 total leucocytes/mm.<sup>3</sup>. Thirty  $\gamma$  PGA, however, increases the leucocyte and granulocyte counts to standard levels and above. This is apparently an optimum dose under the conditions of this test.

Crystalline PGA hexaglutamyl conjugate in doses containing 20  $\gamma$  and 30  $\gamma$  PGA is equivalent in its effect on white cell production to PGA itself. It is also shown that the presence of conjugase inhibitor apparently does not influence the leucocytic response to conjugate

TABLE I

*Comparative Effect of Pteroylglutamic Acid and its Hexaglutamyl Conjugate in the Correction of Sulfasuzidine-Induced Leucopenia in the Rat*

No. of rats	Supplement	Granulocytes <sup>a</sup> /mm. <sup>3</sup>	Total leucocytes <sup>b</sup> /mm. <sup>3</sup>
10	20 $\gamma$ PGA, oral	920 (200-1700)	4050 (2200-6700)
8	30 $\gamma$ PGA, oral	4195 (2250-9600)	7685 (6900-11,950)
8	20 $\gamma$ PGA as crystalline conjugate, oral	740 (550-1300)	5200 (2900-7200)
10	30 $\gamma$ PGA as crystalline conjugate, oral	3680 (2000-7300)	8790 (7050-11,450)
10	30 $\gamma$ PGA as conjugate with inhibitor, oral	2940 (1950-4200)	8640 (6900-11,200)
10	30 $\gamma$ PGA, parenteral	2970 (1800-4550)	8400 (7000-11,200)
8	30 $\gamma$ PGA as crystalline conjugate, parenteral	965 (500-1750)	4570 (3750-5050)
6	40 $\gamma$ PGA as crystalline conjugate, parenteral	3860 (1850-5800)	7400 (5350-9550)
10	None	200 (5-250)	2800 (2100-3000)

<sup>a</sup> Pretreatment values for granulocytes averaged 250 or less/mm.<sup>3</sup>.

<sup>b</sup> Pretreatment values for total leucocytes averaged 3,000 or less/mm.<sup>3</sup>.

since 30  $\gamma$  of PGA conjugate containing inhibitor again caused an increase in total leucocytes and granulocytes equal to that produced by the free vitamin.

Although the quantitative effect on leucocyte production is identical for the three compounds tested, Table II shows a difference in their urinary PGA excretion levels. Data were obtained for the 24-hour period immediately preceding, and for two 24-hour periods after the administration of the test dose. The values for PGA excretion following the administration of PGA and crystalline PGA conjugate are essentially similar and are higher than the value obtained after treatment with an equivalent amount of PGA as conjugate-containing inhibitor. This is evidence that the conjugase inhibitor has an effect on the conjugase systems of the rat which reduces the rate of PGA conjugate cleavage, an effect that apparently has no influence on the utilization of PGA conjugate for leucocyte maturation and production.

When 30  $\gamma$  PGA is administered parenterally, granulocytes and leucocytes increase to standard levels or above (Table I)—levels similar to those obtained when 30  $\gamma$  of the vitamin is given orally.

TABLE II

*Urinary Excretion of Pteroylglutamic Acid when the Vitamin or its Hexaglutamyl Conjugate is Administered to Rats with Sulfasuxidine-Induced Leucopenia*

Experiment no.	No. of rats	Day	Supplement	Average urinary PGA excretion $\gamma/24$ hrs.
I	5	1	None	0.09
		2	30 $\gamma$ PGA, oral	2.80
		3	None	0.11
II	6	1	None	0.08
		2	30 $\gamma$ PGA as crystalline conjugate, oral	3.20
		3	None	0.12
III	5	1	None	0.09
		2	30 $\gamma$ PGA as conjugate with inhibitor, oral	1.60
		3	None	0.04

Thirty  $\gamma$  of the vitamin as conjugate when administered parenterally, however, is apparently not a sufficient amount to produce and maintain standard white blood cell levels four days after therapy, although some increase in the number of cells is shown.

Higher levels of conjugate administration, 40  $\gamma$  PGA as conjugate, are effective in producing normal cell counts. This is some evidence that PGA conjugate when given parenterally has a lower activity for white cell production than the free vitamin itself, although the same activity was shown on oral administration, and suggests the possibility that PGA conjugate is modified in the gastrointestinal tract.

### SUMMARY

In the rat with sulfasuxidine-induced leucopenia, crystalline PGA hexaglutamyl conjugate increases total leucocyte and granulocyte production. The conjugate is as active a hematopoietic agent as the free vitamin, pteroylglutamic acid, when administered orally, but it is perhaps slightly less effective on an equivalent basis when injected. The presence of PGA conjugase inhibitor does not influence the utilization of PGA conjugate for leucocyte maturation.

The excretion levels of PGA are similar following the administration of PGA or crystalline conjugate and are higher than the value obtained when conjugate containing PGA conjugase inhibitor is given.

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# The Relationship of Protein Reserves to the Production of Hyaluronidase and Antihyaluronidase

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Received September 10, 1947

## INTRODUCTION

Recent studies have shown that severe protein deficiency brought about by a prolonged low protein diet leads to a marked decrease in the production of antibodies, serum protein, and hemoglobin; and that the feeding of a high quality protein rapidly reestablishes the normal rates of synthesis (1, 2, 3, 4, 5).

The effect of protein depletion and repletion upon the production of enzymes has not been described previously.<sup>1</sup> Hyaluronidase and antihyaluronidase were selected for this study; the former, because of its important relationship to fertility, and the latter, because of its possible importance as a natural defense mechanism.

## GENERAL PROCEDURES

Young adult white male albino rats of the Sprague-Dawley Strain were used. A description of the methods and rations which we have employed for producing protein depletion has been given elsewhere and will not be described in detail (3, 5).

The animals were kept in small groups in large wire-bottomed cages during the depletion period. Those to be depleted of protein were fed a low protein ration, called 4E. The amount of ration fed to each rat each day was 15 g. Simultaneously, comparable control rats were fed a similar ration (4C) adequate in protein and in equivalent amounts. The depletion period lasted 56 days. The rats on the 4E ration were then divided into two comparable groups. This grouping was based upon initial weights, per cent weight loss, and concentration of serum protein and hemoglobin. All animals were placed in individual cages so that the daily food consumption could be measured. The rats to be repleted with protein were fed a diet (I-C) containing a 50/50

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<sup>1</sup> The effect of protein depletion upon succinic dehydrogenase, phosphatase, and cocarboxylase has been studied by Dr. E. P. Benditt, and will be reported at a later date.

mixture of lactalbumin and casein, fed at a 9% protein level ( $N \times 6.25$ ). The other dietary ingredients of the ration were corrected to keep the repletion ration isocaloric with the basal (4E) and control (4C) rations, and to maintain constant levels of fat, roughage, and water. No attempt was made to correct for the vitamins and minerals present in the added protein substance. These were assumed to be at optimal level in the basal and control rations, so that any additional quantities which might be introduced would not be expected to exert an additive effect.

At the end of a 14 day repletion period, the animals were sacrificed; the testes were removed, their capsules stripped, and the decapsulated testes weighed. In each instance the right testis was weighed as a whole, and a portion (390 mg.) was removed from the left testis (Table I). All were boxed individually, labeled, and placed in the

TABLE I  
*Comparison of Values Obtained by Assay of Hyaluronidase in the  
Testes of Normal, Repleted, and Depleted Rats<sup>a</sup>*

	Control	Repleted	Depleted
1. Right Testes			
No. of animals	10	8	8
Testicle wt., mg.	1447 $\pm$ 30	1042 $\pm$ 31	601 $\pm$ 46
$R_0$ (half life), sec.	265 $\pm$ 8	503 $\pm$ 45	1249 $\pm$ 251
A/mg. testis	0.0132 $\pm$ 0.0004	0.0101 $\pm$ 0.0007	0.0079 $\pm$ 0.0008
Protein, mg.	120 $\pm$ 3	93 $\pm$ 4	65 $\pm$ 5
Per cent protein <sup>b</sup>	8.3 $\pm$ 0.1	9.0 $\pm$ 0.2	10.9 $\pm$ 0.4
A/mg. protein	0.159 $\pm$ 0.005	0.113 $\pm$ 0.007	0.074 $\pm$ 0.008
2. Left Testes (390 mg.)			
No. of animals	10	8	7
$R_0$ (half life), sec.	1383 $\pm$ 47	1594 $\pm$ 103	1977 $\pm$ 199
A/mg. testis	0.0095 $\pm$ 0.0004	0.0086 $\pm$ 0.0007	0.0071 $\pm$ 0.0005
Protein, mg. <sup>b</sup>	32 $\pm$ 1	34 $\pm$ 1	42 $\pm$ 2
Per cent protein <sup>b</sup>	8.3 $\pm$ 0.1	8.9 $\pm$ 0.3	10.7 $\pm$ 0.5
A/mg. protein	0.114 $\pm$ 0.005	0.096 $\pm$ 0.008	0.067 $\pm$ 0.007

<sup>a</sup> The values given are the means and, following each, the standard error.

<sup>b</sup> The protein content/mg. testes was greatest in depleted animals. This finding confirmed our previously unsubstantiated belief that the fluid content of depleted testes was less than that of repleted or control testes.

freezing unit at  $-15^\circ\text{C}$ . Four ml. of heart's blood were aspirated from each rat. These samples were defibrinated, centrifuged, and the supernatant serum pipetted off. They were stored at  $4^\circ\text{C}$ ., and their antihyaluronidase concentrations measured the same day.

### Experiment 1

#### *The Influence of Protein Depletion and Repletion upon the Production of Hyaluronidase in the Testes of Adult White Rats*

The existence of a "spreading factor" in mammalian testes was first noted by Hoffman and Duran-Reynals (6), and McClean (7). Meyer and Palmer (8) isolated hyaluronic acid from vitreous humor and umbilical cord. Substances effecting hydrolysis of this mucopolysaccharide were termed hyaluronidases. Chain and Duthie (9) proved that relatively pure preparations of testicular spreading factor possessed hyaluronidase activity. The existing evidence indicates that the factor responsible for the diffusion in the dermis is identical with that exhibiting hyaluronidase activity *in vitro*. The role of hyaluronidase in fertilization was obscure until McClean and Rowlands (10) demonstrated the enzyme to be capable of dispersing the follicle cells surrounding the mammalian ovum. These follicle cells are embedded in a gel (hyaluronic acid ?) which is liquified by the enzyme. The ovum is unaffected. This observation was the stimulus for the subsequent experiments of Rowlands (11), Joel and Eichenberger (12), and others (13, 14), who have shown that normal fertilization requires adequate concentrations of hyaluronidase in the semen.

### EXPERIMENTAL PROCEDURES

1. The substrate (hyaluronic acid) was extracted from human umbilical cords according to the procedure described by Hass (15).

2. A method for measuring quantitatively the fall in viscosity due to the depolymerization of hyaluronic acid was used for the assay of testicular hyaluronidase. The procedure and method of calculating results from viscosimetric data are described by Hass (15), but since slight modification of his technique is necessary when fresh tissue homogenates are used, a brief description of the experimental conditions will be given.

*Assay of Hyaluronidase.* The testes were allowed to thaw at room temperature and were then homogenized in 5 ml. of distilled water for 4 minutes. The homogenate was centrifuged. One ml. of the supernatant, buffered by borate, and 2 ml. of the polysaccharide, buffered by phosphate, were incubated separately at 31°C. After combination of the two solutions, the depolymerization of the polysaccharide was measured at frequent intervals by following the decrease in viscosity.

The decrease of viscosity as a function of time in a representative assay is shown in Fig. 1. The time required to diminish the viscosity of the polysaccharide to 50% of the original level (half-life time =  $R_0$ ) was determined by interpolation of the graph in Fig. 1. Since the initial and  $R_0$  (50%) concentrations of substrate were the same for all assays, the figure ( $A$ ) obtained by computing  $1/R_0 \times 10^5$  is a function of the velocity coefficient. Then

$$\frac{A \text{ (Activity)} \times 5 \text{ (ml. of homogenate)}}{\text{mg. of testicular tissue}} = A/\text{mg. testicular tissue (Table I).}$$

3. The total nitrogen and non-protein nitrogen contents of the homogenates were measured by Kjeldahl distillation and titration. The total protein and per cent protein were computed. From these figures, and those obtained by the computation of activity, the activity/mg. protein was obtained (Table I).

### Experiment 2

#### *The Influence of Protein Depletion and Repletion upon the Production of Antihyaluronidase in the Serum of Adult White Rats*

The presence of a hyaluronidase inhibitor in the blood of animals was first reported by McClean (16). An appreciable amount of this inhibitor substance was found in normal rat serum by Leonard and Kurzrok (17). Hass (15) believes this hyaluronidase inhibitor to be an enzyme (Anti-Invasin I). The proof of its enzymatic nature was based upon study of its properties, the kinetics of its reaction, and upon its differentiation from antibodies. It was found in the serum of fish, birds, and mammals (in-

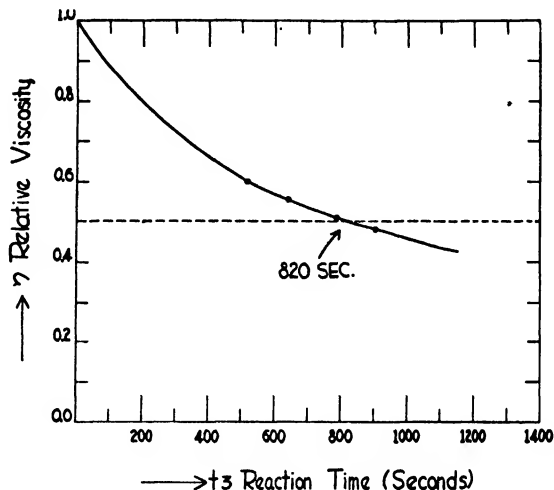


FIG. 1. Depolymerization of the polysaccharide as a function of time.  
Exp. I. 1 ml. of homogenate (rt. testis—rat 78-6).

cluding man). Its reaction was found to be nonspecific as it reacted with about equal velocity with hyaluronidase from a number of sources.

It has been pointed out by Duran-Reynals (18) that the invasiveness of pathogenic bacteria parallels the ability to secrete spreading factor. Invasiveness and virulence are not synonymous. Many pathogenic bacteria are not invasive but are capable of bringing about rapid death of the host. Antihyaluronidase, therefore, can be of importance only in so far as it alters the invasiveness of organisms, and in this respect may be of importance as a natural defense mechanism.

### EXPERIMENTAL PROCEDURES

1. Hyaluronidase was prepared from bovine testes and purified according to the procedure of Madinaveitia (19).

2. Antihyaluronidase destroys hyaluronidase. Its activity and concentration can be determined quantitatively by measuring hyaluronidase destruction in a given time. The procedure and method of calculating results are described by Hass (15).

## DISCUSSION

The results of Experiment I are tabulated in Table I, and those of Experiment II in Table II.

TABLE II

*Comparison of the Values Obtained by Assay of Antihyaluronidase in the Serum of Normal, Repleted, and Depleted Rats<sup>a</sup>*

	Controls	Repleted	Depleted
Number of animals	10	8	8
Activity of antihyaluronidase	2.43±0.10	2.60±0.10	1.71±0.04
Per cent destruction of hyaluronidase	70.6±0.8	72.0±0.8	63.0±0.6

<sup>a</sup> The values given are the means and, following each, the standard error.

I. An analysis of the data presented in Table I conclusively demonstrates that prolonged protein depletion depresses the production of hyaluronidase in the testes of the adult white rat. This depression is both qualitative and quantitative. There is a measurable decrease of hyaluronidase activity per mg. of protein. This diminution of enzyme fabrication is reversible. The feeding of a high quality protein rapidly restores the hyaluronidase. Decreased production of testicular hyaluronidase may be a cause of sterility in undernourished persons who have subsisted for long periods of time on protein-deficient diets.

II. The data summarized in Table II indicate that there is a greater concentration of antihyaluronidase in the serum of normal and protein-repleted rats than in the serum of protein-depleted rats. Benditt, Straube and Humphreys (4) have shown that the plasma volume in control rats is twice that of protein-depleted rats. If this observation is applied to our figures, the difference is magnified. The serum of protein-repleted animals contained, on the average, the greatest amount of antihyaluronidase per unit volume. This may indicate an increased rate of production of the enzyme during the period of repletion of protein-depleted rats.

Experiments in this laboratory have demonstrated that prolonged protein depletion depresses the production of antibodies in the adult rat, and that this depression is reversible (1, 2, 3, 5). This experiment shows that serum antihyaluronidase is similarly affected, and suggests an additional factor predisposing malnourished and starving persons to infection.

## SUMMARY AND CONCLUSIONS

The abilities of protein deficient adult white rats to produce testicular hyaluronidase and serum antihyaluronidase were compared with those of similar animals which had been supplied with high quality dietary protein for 14 days, and with normal control animals.

1. The hyaluronidase activity of testicular homogenate was measured by the viscosimetric method. The testes of protein-depleted rats contained less hyaluronidase than did those of normal controls. Protein repletion of protein-depleted rats by the feeding of a high quality protein produced a rapid increase in the fabrication of the enzyme.

2. The concentration of antihyaluronidase in rat serum was determined by measuring destruction of bovine hyaluronidase. The serum concentration of antihyaluronidase in protein-depleted animals was less than that in the serum of normal control animals. Protein repletion of protein-depleted animals by the feeding of a high quality protein produced a marked increase in the concentration of the enzyme.

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# Malic Dehydrogenase and Cytochrome Oxidase of Lutein and Other Ovarian Tissues During Pregnancy and Lactation<sup>1</sup>

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Received September 12, 1947

## INTRODUCTION

It was shown in a previous report from this laboratory by Meyer, McShan and Erway (1) that the ovaries of pregnant and pseudopregnant rats were high in succinic dehydrogenase and that this high activity was due to the lutein tissue rather than the other ovarian tissues. Later the succinic dehydrogenase of these tissues was studied systematically throughout pregnancy and lactation by Meyer *et al.* (2), and it was shown that the changes in the activity of this enzyme in the lutein tissue could be correlated with the functional changes of this tissue. In the light of these results it was of interest to study the changes in the malic dehydrogenase (MDH-ase) of these ovarian tissues during pregnancy and lactation since both these dehydrogenases catalyze important reactions in the aerobic oxidative cycle. The malic system requires the presence of coenzyme I in order to react with the cytochrome system, but this factor is not necessary for succinic dehydrogenase. As there is this difference between the two systems, and since the cytochrome system is necessary for both enzymes, it was of interest to determine the changes in the activity of cytochrome oxidase at the same time the malic dehydrogenase was being studied. It might be expected that the activities of the two dehydrogenases in a particular tissue would parallel each other since they both function in the aerobic cycle. The results presented in this report show, however, that this is not true for the MDH-ase of the corpora of pregnancy during the last half of gestation.

<sup>1</sup> Supported in part by the Committee on Research in Endocrinology of the National Research Council, and by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.



## MATERIALS AND METHODS

Sprague-Dawley rats 3-4 months of age were used in this investigation. Males were introduced into cages containing females on the day preceding the next estimated estrus. The females were examined the following day to determine whether insemination had taken place. The day following insemination was considered the first day of pregnancy and the day following parturition was designated the first day of lactation. The number of sucklings was reduced to 6 in all cases. The animals were killed by decapitation on days 4, 7, 11, 15 and 20 of pregnancy and lactation, and during the second day of diestrus. Malic dehydrogenase determinations were made on various levels of ovarian and lutein tissues to ascertain whether the oxygen uptake was proportional to the amount of tissue reacting. For these determinations ovaries from pregnant and nonpregnant, and corpora from pregnant rats were used.

Immediately after death the ovaries were removed and the corpora of pregnancy during pregnancy and lactation, and the corpora of lactation during lactation were dissected out as rapidly as possible from the remaining ovarian tissue (ovarian residue). Then each kind of tissue was placed in a moist chamber surrounded by ice, and as soon as all tissues were collected, each kind was weighed and placed in separate homogenizing tubes containing 0.1 ml. of glass-distilled water. The homogenizing tubes that contained the tissues were kept in an ice bath during homogenization and until the homogenates were placed in the Warburg flasks.

During lactation the corpora of pregnancy were distinguished from those of lactation as the latter were lighter in color, had more prominent ovulatory papillae, and were larger after day 7 of lactation than those of pregnancy. The corpora lutea from all rats killed on any particular day of pregnancy or lactation were combined and weighed. All the corpora collected on a given day were counted and the average weight of the corpora for that day was obtained by dividing the total weight by the number of corpora. The ovarian residues remaining after dissection of the corpora lutea were combined, weighed, and prepared for testing.

The method of Potter (3) was used for determining the malic dehydrogenase in the ovarian tissues. Two and one-half per cent glass-distilled water homogenates were made of the three tissues and 0.4 ml. of each homogenate, which was equivalent to 10 mg. of fresh tissue, was placed as soon as possible after homogenization in the proper Warburg flask. Conventional Warburg flasks without side arms were used. The flasks contained 0.3 ml. of 0.5 *M* *l*-malate, 0.6 ml. of 0.5 *M* glutamate, 0.6 ml. of 0.1 *M* nicotinamide, 0.3 ml. of a 0.5% (5 mg./ml.) solution of coenzyme I (diphosphopyridine nucleotide), 0.3 ml. of  $4 \times 10^{-4}$  *M* cytochrome c, 0.4 ml. of 0.2 *M* phosphate buffer of pH 7.4, and 0.1 ml. of glass-distilled water to make a final reaction volume of 3.0 ml. The center wells of the flasks contained 0.1 ml. of 2 *N* NaOH. Studies were made to show that these concentrations of the above reagents were optimum.

Cytochrome c was prepared from beef heart muscle according to the method of Keilin and Hartree (4), except that the final product was dialyzed against glass-distilled water instead of 1% sodium chloride solution. Coenzyme I was prepared according to the LePage (5) modification of the Williamson and Green method (6). Eimer and Amend, and Eastman samples of *l*-malic acid were neutralized with NaOH and used, and values obtained with each were found to be in close agreement.

Approximately 20 minutes elapsed between the time the animals were killed and the placing of the flasks in the Warburg bath, which was at 38°C. The flask contents

were allowed to equilibrate for 6 minutes, then the manometer stopcocks were closed and readings were taken at 5, 10, 20, 30 and 45 minutes. It was found that readings taken at 20 and 30 minutes were, in most cases, the highest and most reproducible values; for this reason  $Q_{O_2}$  values were based on the period from 10 to 30 minutes.

Dry weight determinations were made of the three tissues and were essentially in agreement with results previously obtained by Meyer *et al.* (2). The value of 20% dry weight was chosen as the basis for the calculation of  $Q_{O_2}$  values for all three tissues.

The MDH-ase is reported as  $Q_{O_2}$ , or mm.<sup>3</sup> of oxygen consumed/mg. of dry tissue/hour. MDH-ase per corpus luteum was determined by multiplying the  $Q_{O_2}$  by the average dry weight of the corpus luteum, and the per cent change in activity is graphed in Fig. 2 using the diestrous corpora as the base.

Cytochrome oxidase determinations were made on corpora of diestrus, and of pregnancy on days 7 and 15 of pregnancy and day 20 of lactation, and on corpora lutea of lactation on days 7 and 20 of lactation. One per cent homogenates of the tissues were prepared and 0.2 ml. and 0.4 ml. of homogenate were used in making each determination. The flasks contained, in addition to the homogenate, 1.0 ml. of 0.1 *M* phosphate buffer of pH 7.4, 0.3 ml. of  $4 \times 10^{-4}$  *M*  $AlCl_3$ , 0.3 ml. of  $9 \times 10^{-4}$  *M* cytochrome c, 0.3 ml. of 0.114 *M* sodium ascorbate of pH 7.0 (added just before the flasks were placed in the bath) and enough glass-distilled water to make a final volume of 3.0 ml. The center wells contained 0.1 ml. of 2 *N* NaOH. The flasks were equilibrated for 6 minutes, readings were taken at 10, 20, 30 and 40 minutes after closing the manometer stopcocks.

The average oxygen uptake based on four 10 minute readings for 0.2 ml. of tissue was subtracted from the average oxygen uptake for 0.4 ml. of tissue and the difference was taken as the corrected oxygen uptake for 0.2 ml. of tissue and was used to calculate the  $Q_{O_2}$ . This method of calculation automatically corrects for the autooxidation of the ascorbic acid, which cannot be corrected for by running a blank without tissue. Dry weight values of 20% were used in making the  $Q_{O_2}$  calculations.

## RESULTS AND DISCUSSION

The data presented in Table I show that the oxygen uptake for MDH-ase of whole diestrous ovaries and of corpora lutea of 11 day pregnant rats is proportional to the amount of tissue homogenate used when the range of tissue is from 7.5 to 12.5 mg.

The MDH-ase of lutein tissue and ovarian residue, and the weight of corpora lutea during pregnancy and lactation are presented in Table II and Fig. 1. The weights of the corpora of pregnancy and of lactation are essentially the same as those obtained by Biddulph, Meyer and McShan (7, 8), Meyer *et al.* (2), and Stafford, McShan and Meyer (9).

The MDH-ase of corpora of pregnancy increases from the diestrous value to a maximum on day 11 of pregnancy, after which there is a decrease in the second half of pregnancy. This is in contrast to the re-

TABLE I

*Malic Dehydrogenase Activity of Rat Ovarian and Lutein Tissues*

Kind of tissue	Qo <sub>2</sub>		
	ml. 2.5% homogenate		
	0.3	0.4	0.5
Non-pregnant ovaries	22.7	21.0	21.2
	24.9	25.1	26.0
	23.1	28.5	24.8
	31.1	25.5	25.0
	24.5	22.1	23.6
Av.	25.3	24.4	24.1
Pregnant C.L. 11th day	50.8	50.1	
	48.9	43.3 46.6	40.8 43.5

sults obtained by Meyer *et al.* (2) with succinic dehydrogenase in which the value reached on the 11th day of pregnancy was maintained until parturition. During the period of rapid growth of lutein tissue following day 11 of pregnancy the concentration of succinic dehydrogenase per unit weight of tissue remains at the high level found on day 11, but the concentration of MDH-ase per unit weight of tissue decreases during this period of rapid growth. The MDH-ase continues to decrease through most of lactation, but there is a significant rise on day 20 of lactation, at which time the value almost reaches the high level found during pregnancy.

The MDH-ase of corpora of lactation increases gradually from a value of 26.9 at day 4, which is comparable to the value for diestrous corpora, to a maximum value at day 20 of lactation.

The MDH-ase of the ovarian residue remains constant throughout pregnancy and until day 20 of lactation. This is in contrast to the succinic dehydrogenase which decreases markedly on days 4 and 20 of lactation.

The graphs of Fig. 2 represent the percentage change in weights of both types of corpora and also give the per cent change in the total amount of MDH-ase *per corpus* throughout pregnancy and lactation.

TABLE II

*Malic Dehydrogenase Activity of Lutein Tissue and Ovarian Residue and Weight of Corpus Luteum During Pregnancy and Lactation*

Stage	No. of expts.	Malic dehydrogenase activity (QO <sub>2</sub> )			Weight (mg.)	
		Corpora of		Ovarian residue	Corpora of	
		Pregnancy	Lactation		Pregnancy	Lactation
Diestrus 2nd day	5	26.0 (24.6-27.6)		22.4 (18.7-25.5)	1.0 (0.9-1.1)	
Pregnancy days						
4	5	31.9 (23.4-36.8)		23.5 (16.7-28.4)	1.4 (1.2-1.6)	
7	5	37.4 (30.0-50.0)		23.9 (18.4-32.6)	1.6 (1.2-1.6)	
11	5	44.8 (38.4-50.8)		23.4 (20.9-25.8)	2.20 (2.0-2.3)	
15	5	35.7 (33.6-39.6)		22.6 (19.0-25.2)	4.0 (3.8-4.4)	
20	6	31.0 (25.8-40.3)		23.6 (19.6-32.0)	4.4 (3.9-5.1)	
Lactation days						
4	5	27.2 (21.8-33.9)	26.9 (22.6-30.0)	23.7 (15.1-31.7)	2.4 (2.2-2.5)	1.4 (1.3-1.5)
7	6	23.1 (13.8-31.8)	31.0 (18.4-35.8)	20.7 (10.4-27.8)	1.4 (1.3-1.8)	1.70 (1.6-1.8)
11	5	29.3 (26.6-33.9)	37.4 (26.7-44.2)	21.7 (17.0-26.0)	0.9 (0.7-1.2)	1.9 (1.7-2.0)
15	5	26.0 (23.1-34.7)	42.6 (36.3-55.9)	20.8 (17.5-24.2)	0.60 (0.3-0.7)	1.9 (1.8-2.1)
20	5	40.0 (30.6-57.8)	55.3 (44.2-61.7)	29.5 (26.0-36.4)	0.63 (0.5-0.8)	1.8 (1.5-2.0)

As can be seen from the graphs the highest level of MDH-ase in the ovary is present in the corpora on day 15 of pregnancy.

In Table III data are presented on the cytochrome oxidase activity of corpora of rats in diestrus and at two points each during pregnancy and lactation. Corpora lutea of pregnancy are high at day 7 of preg-

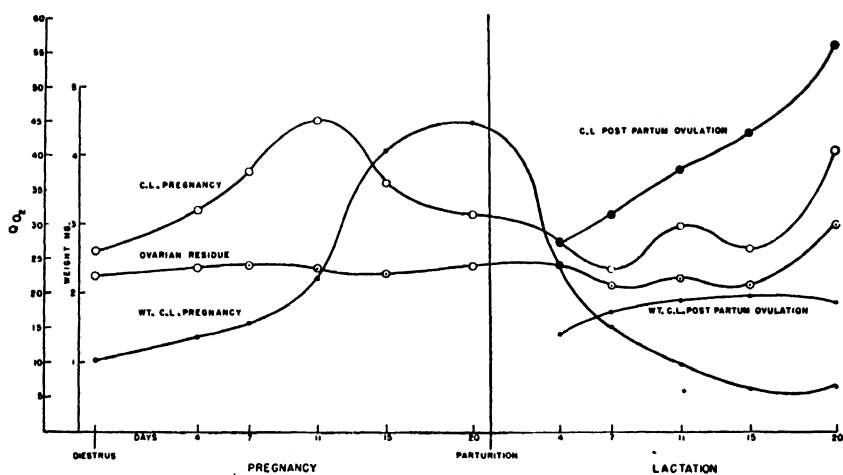


FIG. 1. The malic dehydrogenase and weight of the corpora lutea of pregnancy during pregnancy and lactation, the malic dehydrogenase and weight of the corpora of the post-partum ovulation during lactation, and the malic dehydrogenase of the ovarian residue during pregnancy and lactation.

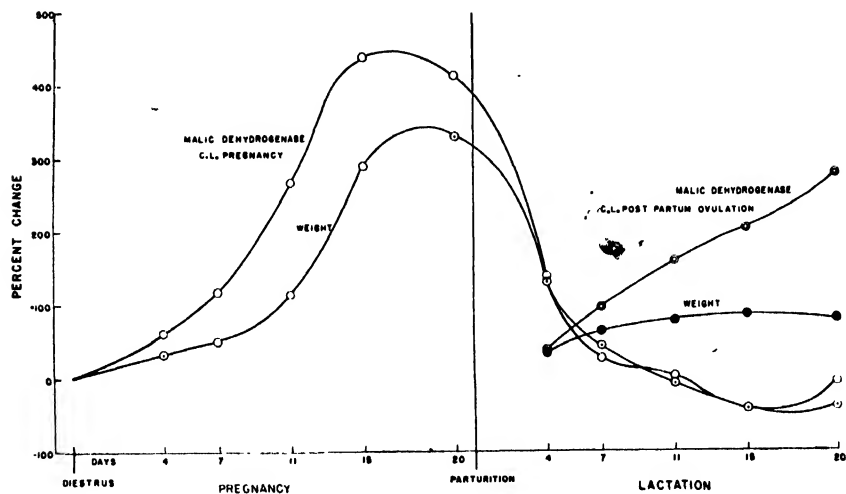


FIG. 2. Percentage change in the malic dehydrogenase and weight of the corpora lutea of pregnancy during pregnancy and lactation, and the percentage change in the enzyme and weight of the corpora of the post-partum ovulation during lactation.

TABLE III

*Cytochrome Oxidase Activity of Corpora Lutea during Pregnancy and Lactation*

Condition	Corpora of pregnancy	Corpora of lactation
Diestrus	248.2	
	265.9	
Av.	257.1	
7th day pregnancy	368.2	
	403.0	
Av.	385.6	
15th day pregnancy	345.0	
	368.1	
	448.9	
Av.	387.4	
7th day lactation	274.5	374.4
	328.5	303.3
Av.	301.5	338.8
20th day lactation	221.8	382.2
	245.5	457.2
Av.	233.6	419.7

nancy, maintain the value at 15 days, decrease at day 7 of lactation and return to the low diestrous value on day 20 of lactation. The highest level of activity is reached by the corpora of lactation at day 20 of lactation and this value is 60% higher than the diestrous value.

The cytochrome oxidase values presented in this paper show the same trend during pregnancy and lactation as those reported by Meyer *et al.* (2). The absolute values presented here, however, are lower than the values reported previously. This is due to the fact that the values reported in this paper were corrected for the autoxidation of the ascorbic acid used as substrate for cytochrome oxidase.

Changes in the activity of enzymes are believed to be related to the processes concerned with growth and function of tissues; particularly is this true for enzymes that make energy available for processes such as tissue growth and function. Results which have been reported seem to support this concept. Potter, Schneider and Liebl (10) showed that the activities of succinic dehydrogenase, cytochrome oxidase and

adenosine triphosphatase of brain tissue of rats increase markedly during the postnatal period beginning 6 days after birth. The activities of these enzymes in the liver increase rapidly during late embryonic life and reach adult levels 10–15 days after birth. It was pointed out that these enzymes represent both energy-yielding and energy-depleting types, and that the potential rate of energy mobilization became greater as indicated by the increase in both types of enzymes during the period of increasing function and increased differentiation.

Other workers have reported results on the relation of the activity of certain enzymes to the growth and functional activity of various tissues. Flexner and Flexner (11) showed that, in early fetal life, the succinic dehydrogenase of the cerebral cortex is approximately 35% of the adult level, and an increase begins around 70 days and reaches the adult level at birth. Friedenwald, Herrmann and Buka' (12) found about 90% of the cytochrome oxidase, succinic dehydrogenase and fumarase of the choroid plexus in the epithelium and 10% in the stroma. The activities of malic and lactic dehydrogenases were as great per cell in the stroma as in the epithelium. Chepinoga (13) found that the activity of succinic dehydrogenase was 50–100% higher in pulp taken from trained muscle than from pulp taken from normal muscle, and there was a decrease in activity in pulp taken from fatigued muscle. Raska (14) found a significant increase in the activities of cytochrome oxidase and succinic dehydrogenase and in the concentration of cytochrome c in the kidney of dogs whose other kidney had been removed; these increases precede hypertrophy of the kidney. There were marked decreases in the activities of these enzymes and in the concentration of cytochrome c in slices and suspensions of kidney from hypertensive dogs. In this connection it is of interest to point out that malic dehydrogenase and cytochrome oxidase in the corpora lutea of pregnancy reach high levels before the corpus begins to grow rapidly. This was true even to a greater extent for succinic dehydrogenase as reported previously by Meyer *et al.* (2).

The results presented in this report for malic dehydrogenase and cytochrome oxidase also show that changes in activity or concentration are correlated with changes in growth and/or function. Beside the changes in the activity of these enzymes in rat lutein tissue, it has been shown previously that there are significant changes in the activities of succinic dehydrogenase (2), adenosine triphosphatase (7, 8), and acid and alkaline phosphatases (9) of lutein tissue during pregnancy and lactation. In most cases, these increases in enzyme activities are correlated with changes in tissue function, and they are believed to be associated with the energy requirements of the tissue as it fluctuates in function. It is of interest that the activities of the above named enzymes change little in the other tissues of the ovary during these reproductive phases.

## SUMMARY

The oxygen uptake of the malic dehydrogenase system of ovarian and lutein tissue is proportional to the amount of tissue homogenate used.

The malic dehydrogenase activity of corpora lutea of pregnancy increases through the first half of pregnancy, reaching a high level at 11 days, then decreases through the second half of pregnancy and through lactation.

The malic dehydrogenase activity of corpora lutea of lactation increases steadily during lactation, reaching a maximum level at the 20th day.

The malic dehydrogenase activity of ovarian residue remains constant through pregnancy and lactation until the 20th day, at which time there is a slight increase.

The cytochrome oxidase activity of corpora lutea of pregnancy maintains a high value at 7 and 15 days of pregnancy and decreases to a value comparable to diestrus at the 20th day of lactation.

The cytochrome oxidase activity of corpora of lactation increases during lactation and reaches a high level on the 20th day.

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# Synthetic Medium for *Streptomyces griseus* and the Production of Streptomycin

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Received September 17, 1947

## INTRODUCTION

A synthetic medium for the production of streptomycin by *Streptomyces griseus* (Krainsky) Waksman and Henrici, 1943 (1), was investigated because of its basic importance to experimentation, commercial production, and purification. These advantages are in part fulfilled by the chemically defined medium described herein. Beef-extract medium<sup>2</sup> for streptomycin production (2) is objectionable because of the cost and variability of certain constituents. The purpose of this paper is to present the results obtained and to give the formula for the medium.

## MATERIALS AND METHODS

The culture of *S. griseus* was Waksman's strain 9<sup>3</sup> (2, 3).

Estimation of streptomycin was by the paper disc-plate assay method against *Bacillus subtilis* (4).

Growth of *S. griseus* on surface culture was estimated by visual comparison with that on beef-extract liquid medium, evaluated as "excellent," and growth in shaken culture with that on corn steep medium<sup>4</sup> (as "excellent").

Growth conditions were maintained as follows: Culture room temperature 26°C. ( $\pm 1^\circ$ ) and relative humidity 57% ( $\pm 3\%$ ); medium after sterilization between pH

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<sup>1</sup> Supported in part by a grant from Abbott Laboratories, The Eli Lilly Company, Parke Davis and Company, and The Upjohn Company.

<sup>2</sup> Beef-extract 5 g., peptone 5 g., glucose 10 g., sodium chloride 5 g. per liter of distilled water; pH 7.0.

<sup>3</sup> Letter from Waksman to junior author dated September 1, 1944.

<sup>4</sup> Corn steep liquor 6 g., glucose 10 g., peptone 5 g., NaCl 5 g. per liter of distilled water; adjusted to pH 7.0.

6.0 and 7.0; for surface growth an air surface of 1.0 cm.<sup>2</sup>/ml. of liquid 1 cm. deep (25 ml./125 ml. Erlenmeyer flask); and for shaken culture an agitation at 100 cycles per minute on 5.3 cm. horizontal stroke with 100 ml. of medium/500 ml. narrow mouth Erlenmeyer flask (air surface when stationary of 0.7 cm.<sup>2</sup>/ml. of liquid 1.5 cm. deep). Unless otherwise stated, all growth and production data refer to 10-day old surface cultures.

Inoculum for surface culture was a suspension of fragmented mycelial mat and conidia (surface mats of 7–10 day growth on beef-extract medium in 2 quart rectangular milk bottles, beer decanted, one mat of 187 cm.<sup>2</sup> suspended in 100 ml. of sterile distilled water, fragmented in a Waring blender at high speed for 10 seconds). Three to five drops of the suspension were introduced into each flask by pipetting the drops to the glass surface just above the liquid in tilted flasks. This permitted the inoculum to remain on the surface of the medium, a condition necessary for growth. For shaken culture the inoculum consisted of 3 ml. per flask of 36 hour growth in shaken culture on corn steep medium which in turn had been inoculated with the growth from nutrient agar slants obtained from a stored soil culture.

### PRELIMINARY STUDIES

First, optimal temperature and H ion concentration for growth and streptomycin production on beef extract medium were found to be 30°C. and pH 7.0 which, in general, confirms previous reports (2, 5). With the pH constant and temperatures at 5° intervals between 15° and 40°C. (with 37°C. optimum (6) included) growth was "excellent" between 20° and 37°C. and no growth occurred at 40°C. Productions of streptomycin were: 24 u/ml. at 15°, 52 at 20°, 84 at 25°, 249 at 30°, 3 at 35° and none at 37°C. Production was not increased by altering the temperature during cultivation following an initial 3 day growth period at 20°, 25° and 30°C. To prove that no inactivating substance was responsible for the failure of the organism to produce streptomycin at temperatures above 35°C., a measured quantity of streptomycin was added to the original beers, boiled and non-boiled, and incubated at intervals of 1 pH unit from pH 4.5 to pH 8.5 in 0.1 M phosphate buffer at 30° and 37°C. for 4 days. Assays showed no decrease in streptomycin.

Secondly, nutrients for the organism were ascertained by incorporating many substances individually and, to some extent, in combination (inorganic and organic nitrogen, amino acids, vitamins, various cations of mineral and organic acids) into a basal medium consisting of 0.05 M glucose and 0.05 M potassium phosphate buffer at pH 7.0. Response in growth and production occurred from substances containing am-

monium, lactate, magnesium, zinc, iron, manganese, and copper ions. This information guided further studies with a trial medium.<sup>5</sup>

### EXPERIMENTS AND RESULTS

The optimal concentration of each constituent for streptomycin production was determined by combining varied amounts of one constituent with other substances constant at the amounts chosen for the trial medium. Preliminary results have been reported (8). Glucose was varied first and its optimal amount (0.04 molal) was substituted. Although the concentrations selected for the trial medium were not optimal, they were maintained in subsequent experiments to avoid introduced variables from altered concentrations. To vary the amounts of ammonium, lactate, potassium, and phosphate constituents, ammonium nitrate, lactic acid,<sup>6</sup> potassium chloride, and sodium phosphate were substituted at equivalent concentrations for ammonium lactate and potassium phosphate.

The results are summarized in Tables I and II. Growth was *nil* when ammonium nitrate was omitted from the medium and sodium nitrate or sodium nitrite substituted for sources of nitrogen.

The reaction of the medium, in general, became alkaline along with the production of streptomycin. However, in the case of varied amounts of glucose, the H ion concentration depended upon the amount of sugar originally present in the medium. It was acid with high sugar and alkaline with low sugar, irrespective of streptomycin production. It was more alkaline with low amounts of magnesium sulfate than with high concentrations.

To study the effect of calcium carbonate on the responses from magnesium sulfate, the two chemicals were added in varied amounts. The amount of  $\text{CaCO}_3$  altered the range in  $\text{MgSO}_4$  concentration at which "excellent" growth occurred. Maximal  $\text{MgSO}_4$  concentration

<sup>5</sup> Glucose, potassium phosphate buffer at pH 7.0, and ammonium lactate each at 0.05 molal, magnesium sulfate at 0.004 molal, and zinc sulfate, ferrous sulfate, manganous chloride, and cupric sulfate each at  $4 \times 10^{-5}$  molal. The concentrations for Mg, Zn, Fe, Mn, and Cu were deduced from a synthetic medium developed by Ezekiel (7) for another soil microorganism, *Phymatotrichum omnivorum* (Shear) Duggar. Reagent, C. P. and U.S.P. grades of chemicals were employed for expediency.

<sup>6</sup> Sodium hydroxide was used to neutralize the lactic acid. Commercial samples of sodium lactate supplied by the Clinton Co., Clinton, Iowa, and Sheffield Farms Co., Inc., New York, N. Y., were satisfactory.

TABLE I

*Growth in Ten Days of Streptomyces griseus on the Trial Medium with Varied Concentration of Constituents in Surface Culture at 26°C.*

Constituent	Growth without constituent	Molarity concentration		
		Range used	Optimum for growth	Inhibition of growth
Glucose	Moderate	.001 - .5	.06-.2	None
Ammonium (nitrate)	None	.0005-1.0	.005-.1	1.0
(Disodium) phosphate	None	.0001- .5	.005-.2	.5
Potassium (chloride)	Light	.0001-1.0	.001-.1	1.0
(Sodium) lactate	Moderate	.0001-1.0	.005-.5	.7
Magnesium (sulfate)	Light	.0001-1.0	.0005-.005	None
Zinc (sulfate)	Light	$10^{-10}$ - $5 \times 10^{-2}$	.00005-.001	.05
Ferous (sulfate)	Good	$10^{-10}$ - $5 \times 10^{-3}$	.000005-.001	.05
Manganous (chloride)	Excellent	$10^{-10}$ - $5 \times 10^{-2}$	None	.05
Cupric (sulfate)	Excellent	$10^{-10}$ - $5 \times 10^{-4}$	None	.0005

TABLE II

*Effect of Varied Concentration of Constituents in the Trial Synthetic Medium upon Streptomycin Production in Ten Days by Streptomyces griseus in Surface Culture at 26°C.*

Constituent	Production without constituent		Molarity concentration				
			Optimum for production			Production of 50 units or above	
	(Units) <sup>a</sup>	(S.D.%) <sup>b</sup>		(Units) <sup>a</sup>	(S.D.%) <sup>b</sup>	(pH) <sup>c</sup>	
Glucose	36	59	.04	106	23	7.3	.001-.2
Ammonium (nitrate)	0	0	.08	122	20	7.7	.005-.5
(Disodium) phosphate	0	0	.01	102	9	7.2	.001-.02
Potassium (chloride)	17	11	.06	62	9	7.4	.01-.1
(Sodium) lactate	28	8	.1	180	19	8.5	.005-.5
Magnesium (sulfate)	11	10	.01	108	45	6.4	.005-.1
Zinc (sulfate)	10	10	.00005	68	10	7.0	.00005-.001
Ferrous (sulfate)	19	9	.00005	82	18	6.9	.00005 only
Manganous (chloride)	51	9	.0005	93	12	6.5	.00005-.005
Cupric (sulfate)	47	23	None <sup>d</sup>				3 concentrations <sup>e</sup>

<sup>a</sup> Units of streptomycin/ml.

<sup>b</sup> Standard deviation percent of units based on 6 or 9 replications.

<sup>c</sup> Final pH at 10 days.

<sup>d</sup> Production at  $5 \times 10^{-9}$  and  $1 \times 10^{-6}$  molal was reduced to 37 units/ml.

<sup>e</sup> Productions at  $1 \times 10^{-9}$ ,  $5 \times 10^{-8}$  and  $1 \times 10^{-6}$  molal were higher than without copper but were without significant differences.

for 0.1, 0.5, and 3%  $\text{CaCO}_3$  was 0.1 molal  $\text{MgSO}_4$ , 1% was 0.5 molal, 5% was 0.05 molal, and 10% and without  $\text{CaCO}_3$  was 0.005 molal; minimal concentration for 0.1, 0.5, 1, 3, and 5%  $\text{CaCO}_3$  was 0.0001 molal, without  $\text{CaCO}_3$  0.0005 molal. Highest production occurred at 0.05 molal  $\text{MgSO}_4$  with all amounts of  $\text{CaCO}_3$  except for 5 and 10%, at which 0.01 molal was optimal, the same as without  $\text{CaCO}_3$ . Calcium chloride did not affect growth or production as did  $\text{CaCO}_3$ .

Additional salts (potassium chloride, sodium chloride, or sodium sulfate) at varied concentrations from 0.001 to 0.1 molal in the complete medium did not affect streptomycin production or growth. At 0.5 and 1 molal they inhibited growth.

Additional trace elements (Co, Mo, Cr, B, and I), at varied amounts from  $10^{-10}$  to  $10^{-3}$  molal, when added to the complete medium did not affect production or growth, except for chromium and iodine, which inhibited growth at  $10^{-3}$  molal. Upon the addition of a number of trace elements (Co, Mo, As, Hg, Pb, W, Bi, U, Be, Ti, V, Sr, Th, Ba, and Sb) each at  $10^{-5}$  molal in an accumulative series of first Co then Co and Mo, then Co, Mo, and As, *etc.*) to the medium containing Zn, Fe, Mn, and Cu, there was no measurable response in production. Growth appeared to be retarded by Pb, W, Bi, U, Be, Ti, and Ba. When some trace elements were added individually to the medium without Zn, Fe, Mn, and Cu, growth and streptomycin production were affected. The elements tested and the productions obtained are given in Table III.

Utilization of certain nutrients, as determined by chemical analyses,<sup>7</sup> was glucose 97.8%, lactate ion 93.3%, and ammonium nitrogen 50.0%.

Submerged growth in the trial medium yielded from 36 to 102 units of streptomycin/ml. in three days without  $\text{CaCO}_3$ , and from 28 to 68 units with 0.1%  $\text{CaCO}_3$ .

A modified trial medium based upon the concentrations that were found to be optimal in the trial medium (see Col. 4, Table II) was evaluated. With surface culture, growth was "excellent" and production was 206 units/ml. in 10 days. With shaken culture, production was 90 units/ml. in 3 days and growth was "excellent." When glucose was varied, the optimal amount was 0.1 molal for surface culture (426 units/ml.) and 0.2 molal for shaken culture (272 units/ml. in 5 days). The alkalinity of the medium in shaken culture increased with time of

<sup>7</sup> Analyses by Dr. David Pearlman are acknowledged.

TABLE III

*Relative Effect of Trace Elements Individually (Concentration of  $1 \times 10^{-5}$  Molar Number of Respective Atoms in Molecule) without Zinc, Iron, Manganese, and Copper in the Trial Medium on Production of Streptomycin by Streptomyces griseus in Surface Growth for Ten Days at 26°C.*

Trace Elements and Streptomycin Units Per ML.					
Stimulation (21 or more units)		No effect (6-20 units)		Inhibition (0-5 units)	
No CaCO <sub>3</sub>	With CaCO <sub>3</sub>	No CaCO <sub>3</sub>	With CaCO <sub>3</sub>	No CaCO <sub>3</sub>	With CaCO <sub>3</sub>
Mo(ic) 59	Co(ous) 68	Fe(ous) 18	Ag 20	As(ite) 5	As(ite) 0
Mo(ate) 47	Sr 68	V(ate) 18	Be 20	Cr(ate)* 5	B 0
Hg(ic) 41	As(ate) 57	Sr 15	Pb 20	Cu(ic) 0	Ce 0
Bi 38	W 50	Th 14	Cr(ic) 16	Ni 0	F 0
Pb 38	Ba 36	Ba 13	Cr(ate) 16	Sn 0	I 0
As(ic) 36	Ti 36	F 12	Mn(ous) 16		Ni 0
Mn(ous) 36	V(ate) 36	B 11	As(ic) 12		Sb 0
U 33	Zn 31	Cr(ic) 11	Bi 12		Sn 0
Be 31		I 11	Cd 10		Th 0
Zn 31		Sb 8	Cu(ic) 10		
W 28		Ce 7	Fe(ous) 10		
As(ate) 26			Hg(ic) 10		
Co(ous) 26			Mo(ic) 10		
Ti 26			Mo(ate) 10		
			U 10		

Controls without trace elements and CaCO<sub>3</sub> = 10 units.

Controls without trace elements but with 0.1% CaCO<sub>3</sub> = 11 units.

fermentation until it finally reached approximately pH 9.0. The rate of this change was inversely related to the sugar concentration. At 0.05 molal and below, the reaction was pH 8.0, or above, in 3 days, whereas with 0.08, 0.1, 0.2, and 0.3 molal a pH 8.0 was reached in 4, 5, 5, and 8 days, respectively. In general, peak production for any concentration of sugar occurred when the reaction of the medium reached approximately pH 8.0. Sodium chloride at 5% did not affect production, pH, or growth. Ammonium chloride and ammonium sulfate were substituted for ammonium nitrate without any effect on growth or the amount of streptomycin produced in either surface or shaken culture. Ammonium chloride increased the rate of production in shaken culture.

A synthetic medium formulated from these results is as follows:

Glucose for surface culture	0.1	molal	18.0 g.
Glucose for shaken culture	0.2	molal	36.0 g.
Sodium lactate <sup>*</sup>	0.1	molal	11.2 g.
Ammonium chloride	0.08	molal	4.28 g.
Potassium chloride	0.06	molal	4.47 g.
Disodium phosphate (7H <sub>2</sub> O)	0.01	molal	2.68 g.
Magnesium sulfate (7H <sub>2</sub> O)	0.01	molal	2.46 g.
Zinc sulfate(7H <sub>2</sub> O)	0.00005	molal	14.37 mg.
Ferrous sulfate (7H <sub>2</sub> O)	0.00005	molal	13.90 mg.
Manganous sulfate (H <sub>2</sub> O)	0.0005	molal	84.50 mg.
Distilled water			1000. ml.
H ion concentration after sterilization <sup>*</sup>			pH 6.5-7.0

### DISCUSSION

Streptomycin production is not directly related to growth of the organism or invariably associated with alkalinity of the fermented medium. An explanation for the lack of streptomycin in cultures of "excellent" growth at 35 and 37°C. (LaPage (9) also observed retarded production by high summer temperatures) is not apparent. It is of interest, however, that the organism utilizes lactic acid and a lactic acid-forming enzyme is reported to be inactivated at 37°C. (10). In addition sugar oxidation in an alkaline suspension of metal hydrosols containing electrolytes is retarded by such temperatures (11). It is also reported that the production of amino acid decarboxylases by *Escherichia coli* is inhibited at 37°C. (12). The H ion concentration of the fermented medium is associated with the relative concentration of acid and alkaline residue-forming constituents.

Growth and streptomycin production obtained from simple chemicals discredit the claims that streptomycin production largely depends upon the presence of a growth-promoting agent or a certain organic substance as supplied by beef or meat extract (2, 3, 5). Furthermore, contrary to previous claims that this organism (2, 3, 5, 13) and actinomycetes in general (14) utilize nitrates, growth was *nil* when only nitrate nitrogen was available. Growth response from calcium carbonate and not from calcium chloride suggests that this organism

<sup>\*</sup> Lactic acid U.S.P. XII (10.6 g. of 85%/l.) was neutralized with NaOH.

<sup>\*</sup> H ion concentration was adjusted to pH 8.4 with NaOH prior to sterilization in order to obtain a pH 6.5-7.0 in the sterilized medium.



utilizes carbon dioxide as has been reported for *Actinomyces longisporus ruber* on a liquid synthetic medium (15).

The responses from trace elements (reported to act as biocatalysts in oxidation-reduction reactions (16) and by increasing the ionization (17) and oxidation (18, 19) of sugars) indicate that the necessity of these elements for streptomycin production does not necessarily parallel that for growth. Some other reports of trace element requirements for antibiotic production are iron for citrinin (20, 21) and penicillin (22), and manganese for subtilin (23) and tyrothricin (24). The relationship of optimal concentration for any constituent to the overall concentration is not apparent but the total mineral concentration of the synthetic medium (0.16 molal) approaches the reported total optimum (0.12 molal) of three salts for penicillin production (25).

#### SUMMARY

1. The nutrient requirements of *S. griseus* are glucose, potassium, phosphate, magnesium, ammonium nitrogen, lactic acid, and three trace elements (Zn, Fe, and Mn).

2. Ammonium nitrogen and phosphate were essential for growth.

3. Nitrate and nitrite nitrogen were not utilized.

4. Potassium, magnesium, zinc, and iron were necessary for good production and for "excellent" growth.

5. Manganese was not necessary for growth but stimulated production.

6. Lactic acid is utilized and appears to contribute to the production of streptomycin.

7. Calcium was not necessary for growth or production but calcium carbonate affected the responses from magnesium sulfate.

8. The optimal temperature and H ion concentration for streptomycin production are 30°C. and pH 7.0 respectively.

9. At 35° and 37°C. streptomycin was not produced, although growth was "excellent."

10. Each component of the medium had an optimal concentration for production.

11. A synthetic medium for streptomycin production is presented for surface and submerged culture of *S. griseus*, productions being 426 and 272 units/ml., respectively.

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# ***In Vitro* Antibiotic Activity of Crystalline Tomatine<sup>1</sup> Toward *Candida Albicans*. Antagonistic Effect of Rutin and Quercetin**

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Received September 23, 1947

## **INTRODUCTION**

In a recent paper (1) it was reported that rutin is present in dried leaves of the Red Currant tomato (*Lycopersicon pimpinellifolium*) and in concentrates of the antibiotic agent tomatin obtained from these leaves. It was also reported that rutin had no apparent effect on the assay for tomatin activity when the cylinder-plate assay method (2), using *Fusarium oxysporum* f. *lycopersici* (R-5-6) as the test organism, was employed. Crude tomatin concentrates, in general, have been found to be as effective as partially purified tomatin in inhibiting the pathogenic fungi of plants and humans (3, 4). It has now been observed, however, that crude tomatin concentrates incorporated in the culture medium fail to inhibit the growth of two strains of the fungus *Candida albicans*, whereas crystalline "tomatine"<sup>1</sup> (5), recently isolated, and partially purified tomatin (4), completely inhibit these two strains of the organism. Since rutin is a normal constituent of crude tomatin concentrates, it was considered important to investigate the effect of this material and its aglycone, quercetin, on the antibiotic activity of crystalline tomatine toward *Candida albicans* to ascertain whether or not either of these compounds could account for the observed differences in the antibiotic properties of crude tomatin con-

<sup>1</sup> A crystalline compound possessing the antifungal properties of crude tomatin concentrates has been isolated. The crystalline compound has very low antibacterial activity but possesses the antifungal activity of crude preparations and is designated *tomatine* to distinguish it from the crude or partially purified tomatin referred to in earlier publications (3, 4). Crystalline tomatine has been tentatively characterized as a glucosidal alkaloid (5).

centrates and crystalline tomatine. The results of this investigation are reported here.

### EXPERIMENTAL

**Materials.** Rutin and quercetin were obtained from the Eastern Regional Research Laboratory. Crude tomatin concentrate (4) and crystalline tomatine (5) were prepared in this laboratory. A modified medium (pH 6.4-6.6) containing 2.5 g. anhyd. dextrose, 5.0 g. Bacto-yeast extract (dehydrated), 5.0 g. Neopeptone, 15 g. Bacto-agar and distilled water to make 1 l., was used as the basal medium for all experiments.

**Method.** A tomatine solution containing 10 mg./ml. was prepared by first dissolving 10 mg. of crystalline tomatine in 0.1 ml. of 0.1 *N* HCl and then diluting to the required volume. The diluted tomatine solution (pH 3.5) was sterilized and added to the sterile, melted culture medium in the amount desired. The pH of the media remained unchanged, in spite of the addition of the small volumes of slightly acid tomatine solution. Sterile aqueous solutions of rutin and quercetin were prepared and added to sterile, melted culture medium in a similar manner. Slants of the various media were prepared, inoculated with a 2 day old culture of *Candida albicans* (Duke 1036), and incubated at 28°C. for 3 days, after which observations were made.

### RESULTS AND DISCUSSION

The results in Table I show that rutin and quercetin are capable of neutralizing the fungistatic action of tomatine toward *Candida albicans*. The inhibitory effect of 0.1 mg. tomatine/ml. was counteracted by 0.2 mg. rutin/ml. and by 0.05 mg. quercetin/ml. A higher concentration of tomatine (0.25 mg./ml.) required 0.5 mg. quercetin/ml. to give a corresponding antagonistic effect, whereas rutin had no effect even at concentration of 1.0 mg./ml. Rutin and quercetin, *per se*, have no effect on the growth of the test organism, *Candida albicans*.

Rutin is less effective than quercetin in neutralizing the antibiotic activity of tomatine. Approximately 4 times as much rutin (or twice as much on an equivalent weight basis) is required to produce an effect similar to that produced by quercetin. This indicates that the flavonol part of the rutin molecule (quercetin) is responsible for its antagonistic effect toward tomatine.

Rutin has been reported to have no effect on the growth of *Staphylococcus aureus*, whereas its aglycone, quercetin, exhibits considerable toxicity toward this organism (6). Later work has demonstrated that quercetin inhibits the growth of some Gram-positive and Gram-negative organisms but does not effect the growth of certain fungi tested (7). We have found that neither rutin nor quercetin is toxic to *Candida albicans* under our test conditions.

TABLE I

*Antagonistic Effect of Rutin and Quercetin on the Fungistatic Activity of Crystalline Tomatine Toward Candida albicans (Duke 1036)*

(Results expressed as growth, +, or no growth, —, after 3 days incubation at 28°C.)

		Tomatine (mg./ml. medium)				
		0.0	0.01	0.05	0.1	0.25
Rutin (mg./ml. medium)	0.0	++++	+++	++	—	—
	0.01	++++	++++	+++	—	—
	0.05	++++	++++	+++	—	—
	0.1	++++	++++	+++	—	—
	0.2	++++	++++	+++	±	—
	0.5	++++	++++	+++	+	—
	1.0	++++	++++	+++	++	—
Quercetin (mg./ml. medium)	0.0	++++	+++	++	—	—
	0.01	++++	++++	+++	—	—
	0.05	++++	++++	+++	±	—
	0.1	++++	++++	+++	+	—
	0.2	++++	++++	+++	++	—
	0.5	++++	++++	+++	++	+

Rutin is probably only one of the natural constituents of the tomato plant that may be present in, and hence may influence the antibiotic activity of, crude tomatin concentrates. As more plants are investigated it seems likely that the antibiotic activity of crude concentrates obtained from these plants may be influenced in a manner similar to that described for crude tomatin preparations.

#### ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. George W. Irving, Jr., for his interest in this work; to Dr. Norman F. Conant, Duke University School of Medicine, for supplying the culture of *Candida albicans* (Duke 1036); and to Dr. J. F. Couch, Eastern Regional Research Laboratory, for samples of rutin and quercetin.

#### SUMMARY

Crude tomatin concentrates fail to inhibit the growth of the organism *Candida albicans*, whereas crystalline tomatine is a very effective

inhibitor. It has been shown that rutin and quercetin, which normally occur in crude tomatin concentrates, are capable of neutralizing the inhibitory effect of crystalline tomatine toward this organism. Quercetin, the aglycone of rutin, is more effective than rutin in counteracting the inhibitory activity of crystalline tomatine toward *Candida albicans*. The results indicate that the flavonol portion (quercetin) of rutin accounts for its antagonistic property.

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# Maintenance of Carbohydrate Stores before and after Insulin Administration in Rats Prefed Diets Containing Added Glycine<sup>1</sup>

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Received September 30, 1947

## INTRODUCTION

In a recent paper from this laboratory (1) it was pointed out that prefeeding rats on a diet containing added glycine resulted in a marked effect upon carbohydrate storage during an ensuing fast. Rats fed a ration containing 10–15% glycine for 2 days and then fasted for 24 hours had liver glycogen levels of 1–1.3%. These values were 3–4 times those of control rats not receiving glycine. Such a phenomenon has been referred to as the “protein effect” by Mirski and coworkers (2) who first noted increased liver glycogen storage during starvation as a result of prefeeding a diet very high in protein. The term may well be retained and applied also to amino acids having the capacity of bringing about such an alteration in carbohydrate storage. Thus, of the amino acids studied recently (1), only glycine and DL-alanine (slight effect at a high level) were considered to have a “protein effect.” At the levels studied L-leucine and L-glutamic acid did not exhibit a “protein effect.”

It was stated earlier (1) that sufficient data were not at hand to indicate whether the “protein effect” of glycine is due to increased glyconeogenesis, decreased glycolysis or a combination of the two.

Data are presented here which support the hypothesis that this effect of glycine is intimately associated with its capacity to increase the glyconeogenetic processes (under specified conditions). Blood sugar, and liver and muscle glycogen levels have been studied in rats under the stress of a heavy dose of insulin after consuming diets with and without added glycine.

## EXPERIMENTAL

**Rations.** The control ration had the following percentage composition: casein, 16; Wesson salt mixture (3), 5; Brewers' yeast (Squibb), 10; cod liver oil, 2; Wesson oil, 5; commercial white corn dextrin, 54; and glucose, 8.

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<sup>1</sup> Aided by grants from the General Research Council, Oregon State System of Higher Education, and the Diabetic Research Foundation of Portland, Oregon.



The experimental ration contained 15% glycine substituted for an equal weight of dextrin.

*Animals.* Virgin female rats of the Sprague-Dawley strain weighing from 160–200 gs. were used throughout.

*Methods.* The Good, Kramer and Somogyi (4) method was used for both liver and muscle glycogen determinations. The bulk of the gastrocnemius muscle was employed for the latter. Blood sugar was estimated by the micro method of Nelson (5).

*Insulin Administration.* Insulin (Lilly, Iletin) when given was administered at a level of 12 units/kg. subcutaneously at the nape of the neck, after dilution with water to 4 units/cc.

*Plan of a Typical Experiment.* The animals were removed from the Purina Dog Chow stock diet and placed on the control diet for one day to help accustom them to the synthetic rations. They were then fed the glycine diet or kept on the control diet for 36 hours.

At this point, the last meal was given in two parts after dividing the rations into (a) the carbohydrate of the control ration and the carbohydrate plus glycine of the glycine ration, and (b) the remaining constituents. Part (b) was given in the usual manner of feeding and after two hours, when this was generally consumed, part (a) was given by stomach tube. Glucose was substituted for dextrin in both rations to facilitate administration. Identical amounts by weight were given—glucose only for control animals, and glycine plus glucose for glycine-fed rats. This procedure was followed to permit more exact control of the food ingested just prior to fasting. Food consumption in the control animals was adjusted throughout the experiment by pair-feeding with the glycine-fed animals. During these experiments the intake was quite constant and ranged from 14 to 17 gs. per rat for the 38 hours of experimental feeding. This included the food given by gavage.

Eight hours after stomach tubing, blood samples were taken for sugar determinations. Insulin was administered immediately to one half the animals previously given each diet. The remaining animals were sacrificed for muscle and liver glycogen determinations. After another 5 hours, blood samples were again taken from the insulin group and the animals were then sacrificed for liver and muscle glycogen determinations.

## RESULTS

The experiment outlined above was designed to study the effect of prefeeding a diet containing added glycine on the ability of rats to withstand a large dose of insulin after a short fast. This was judged by observing differences in the blood sugar levels, and muscle and liver glycogen values of rats fed diets with and without added glycine, both before and 5 hours after giving insulin. It was felt that such data would aid in establishing the mechanism of the "protein effect" of glycine.

Reference to Table I shows that the data support the hypothesis that glycine in some way stimulates glycogenesis in the rat. It will be seen that the rats fed the control diet had an original muscle glycogen level decidedly lower than the glycine-fed animals. Also, the drop

TABLE I

*The Effect of Prefeeding Extra Glycine on the Response of Rats to 12 Units of Insulin/Kg. as Measured by Changes in Blood Sugar, and Muscle and Liver Glycogen*

	Glycogen (Per cent wet weight)				Blood sugar Mg.-%	
	Control ration		Glycine ration		Control ration	Glycine ration
	Muscle	Liver	Muscle	Liver		
Before insulin	0.43	3.89	0.63	3.51	114	133
After insulin	0.21	0.13	0.52	1.36	66	106
Decrease	0.22	3.76	0.11	2.15	48	27
Per cent decrease	51	96	17	61	42	20

All values represent the average of data from 12 rats except "before insulin" blood sugars (16 rats) and "after insulin" blood sugars (8 rats).

in muscle glycogen as a result of insulin activity, was greater in the former than in the latter animals. The liver glycogen values similarly were manifestly reduced in the control-fed animals as a result of insulin, while the glycine-fed rats maintained carbohydrate in the liver to a high degree. The blood sugar levels of the glycine-fed animals were significantly higher than those of the control-fed rats before insulin administration. It is obvious that recovery from insulin hypoglycemia was far more marked in the glycine-fed rats.

## DISCUSSION

The data in Table I were obtained from three different experiments conducted some weeks apart. In each experiment the data were in surprisingly good agreement.

Absorption of carbohydrate was essentially complete at the time the first blood samples were taken (8 hours after stomach tubing). Total reduction, following acid hydrolysis of the contents of the stomach and small intestine was found to be negligible for both glycine-fed and control-fed rats. Absorption of glycine was also essentially complete at this time.<sup>2</sup>

<sup>2</sup> Credit is due Miss Ellen Talman of this laboratory for conducting the glycine determinations. The method employed was that of Alexander, Landwehr and Seligman (6).

The blood sugar levels 5 hours after insulin are consistent with what might be expected from the liver glycogen values, *i.e.*, hypoglycemia in the control-fed animals with only 0.13% liver glycogen and 106 mg.-% in the glycine-fed animals with 1.36% liver glycogen.

The higher blood sugar levels obtaining before insulin administration in the rats prefed the glycine diet will be considered at a later time. Further data not reported here indicate that this difference is highly significant.

It should be kept in mind that, during the entire feeding period (including stomach tubing), the control animals received more carbohydrate than did those consuming the glycine-fortified ration.

It is of interest to consider the total carbohydrate content, at 5 hours after insulin, of rats prefed the glycine diet compared with those prefed the control ration. Although exact calculations of such values are impossible from the data available, it would appear that the glycine-fed rats contain over twice as much total carbohydrate at this time. The problem, then, is to account for this excess carbohydrate.

The fact that the post-insulin blood sugar levels in the glycine-fed animals are considerably higher than in the control-fed animals appears to rule out the possibility of reduced glycogenolysis in the glycine-fed animals as a factor in accounting for the increased carbohydrate reserves.

Conceivably, the extra carbohydrate content of the glycine-fed rats might have arisen from conversion of glycine stored as such or as a constituent of tissue proteins. However, there is no evidence at present that sufficient glycine storage for this purpose is possible. There also is no evidence to indicate that glycine may be stored in a form that would be available for glycconeogenesis to an unusual degree. On the other hand, there is good evidence that glycine is a relatively inefficient glycogen former (7). It is not comparable to glucose or to alanine in the degree to which it affords protection against lethal doses of insulin, when given by stomach tube at the time of the insulin injection (8), in spite of the fact that its rate of absorption is nearly equal to that of alanine (9).

For analogous reasons it appears unlikely that the results can be explained by storage of glycconeogenetic amino acids derived from glycine, or by a sparing action of glycine on carbohydrate.

No attempt has been made to rule out all possibilities which may account for the data presented. A number of important papers on the

relation of glycine administration to glycogen formation in animals have not been discussed. The reason for this is that the technique invariably involved starvation, administration of the amino acid by itself, and the determination of glycogen some time later. The procedure employed here and in an earlier paper (1) is significantly different, *i.e.*, the amino acid was fed as part of the ration, the animals fasted and then determinations of glycogen, *etc.*, were carried out.

The likely assumption still remains that prefeeding glycine stimulates the general process of glyconeogenesis and that this process becomes more active under the stress of a large dose of insulin. The results are most easily explained at the present by assuming increased adrenal cortical activity.

### SUMMARY

The effects of a heavy dose of insulin on various carbohydrate stores have been studied in rats prefed diets with and without added glycine.

Before the administration of insulin, the glycine-fed animals had higher muscle glycogen and blood sugar levels than the rats which had consumed the control diet. The liver glycogen values were similar.

Five hours after the administration of insulin, the glycine-fed rats showed 2.5 times as much muscle glycogen, over 10 times as much liver glycogen, and a far less pronounced depression of blood sugar, than the animals which had consumed the control ration.

Mechanisms which might account for the increased carbohydrate stores under the influence of insulin in the glycine-fed animals are discussed. It is concluded that the most likely explanation involves an increase in the glyconeogenetic processes as a result of extra glycine ingestion.

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# A New Specific Color Reaction of Galacturonic Acid<sup>1</sup>

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Received October 2, 1947

## INTRODUCTION

In an earlier paper a modification of the general color reaction of carbohydrates with sulfhydryl compounds was described (1), which seems specific for glucuronic acid. The present report deals with another modification of this general reaction, which appears to be specific for galacturonic acid.

## EXPERIMENTAL

### CYSTEINE REACTION OF GALACTURONIC ACID

#### A. Simple Qualitative Test

(a). *Procedure.* A test tube (16 × 150 mm.) containing 1 cc. of an 0.08% solution of galacturonic acid is placed in a water bath at 22–25°C., 4 cc. of conc. H<sub>2</sub>SO<sub>4</sub> are added, the mixture shaken and left in the water until cooled off. One-tenth cc. of a 2.5% solution of cysteine is added and the reaction mixture left at room temperature for 24 hrs. A green-blue color appears and increases in intensity for another 24 hrs.

(b). *Specificity of Reaction.* Pectic acid and *Pneumococcus* polysaccharide T.I give the reaction at the same equivalent concentration, although the color with the second compound is not green blue, but pure blue and more intense. With glucuronic, hyaluronic, chondroitinsulphuric, and alginic acids, with 1-mentholglucuronide and *Pneumococcus* polysaccharide T.III,<sup>2</sup> the reaction is completely negative even at a concentration equivalent to 0.160% of glucuronic acid. All these compounds give yellow, brown and pink colors. Hexoses give an intensive greenish-yellow color and pentoses a pink color.

(c). *Influence of the Concentration of Galacturonic Acid on the Reaction.* In comparing the cysteine reaction of various compounds it is imperative to use equivalent amounts

<sup>1</sup> This work was supported by a grant from the Donner Foundation, Cancer Research Division.

<sup>2</sup> I am greatly indebted to Dr. Karl Meyer for preparations of hyaluronic and chondroitinsulphuric acids; to Dr. Michael Heidelberger for preparations of *Pneumococcus* polysaccharides and Dr. Leonard Cretcher for a preparation of alginic acid.

in every case and the concentration of the hexuronic acid must not be less than 0.08%. The reason for this is that not only the intensity, but also the character, of the color is dependent upon the concentration of the galacturonic acid. Thus, no green or blue color is obtained with solutions containing less than 0.04% of the galacturonic acid. The more concentrated the solution the more the blue color predominates.

### B. Spectrophotometric Test for Galacturonic Acid

When galacturonic acid is present in a solution with an excess of glucuronic or mannuronic acid, free or conjugated, the noncharacteristic

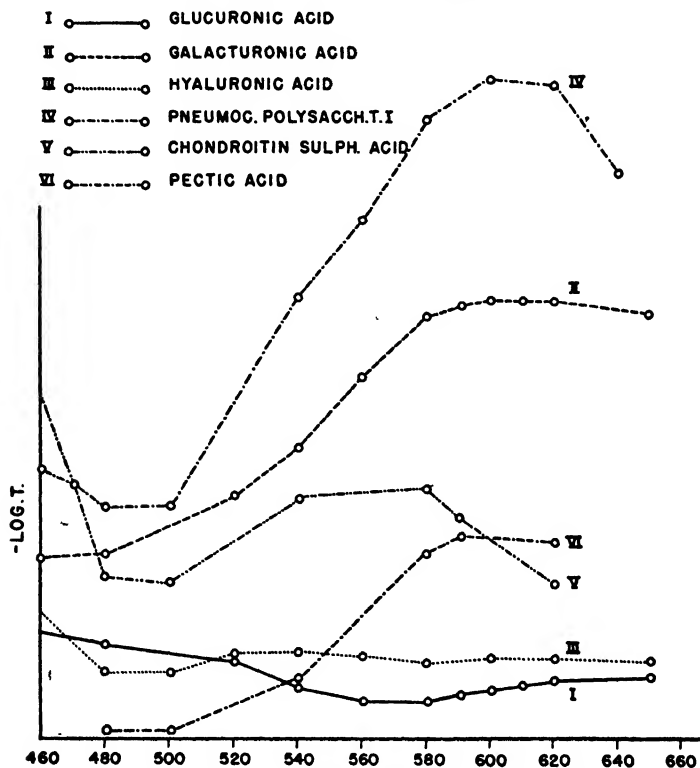


FIG. 1. Absorption spectra of the cysteine reaction of hexuronic acids and polyuronides.

colors produced by the latter will obscure the cysteine reaction of galacturonic acid. Even in this case, however, it is possible to detect galacturonic acid with certainty by spectrophotometric measurements and a slightly more complicated compensation procedure.

(a) *Absorption Spectra.*<sup>3</sup> Galacturonic acid, and polyuronides in general, give non-characteristic yellow, pink and brown colors with  $\text{H}_2\text{SO}_4$  alone. To obtain characteristic absorption spectra of the reaction due to cysteine it is necessary to subtract the absorption due to the reaction with  $\text{H}_2\text{SO}_4$  alone from the total absorption of the sample. This is done by preparing separately from the sample containing the hexuronic acid solution to which cysteine is added, two blanks without cysteine, (1) prepared with water only, and (2) with the solution. Blank (1) is used as the zero point and the density of Blank 2 is subtracted from the total density of the sample with cysteine. As can be seen from Fig. 1, the absorption spectra of galacturonic and pectic acid and of T.I polysaccharide differ considerably from those of glucuronic acid and polyuronides in that part of the spectrum between 5,400 and 6,000 Å. While the spectrum of the latter compounds is practically parallel to the abscissa in this region, those of galacturonic acid and polymers show a steep rise between 5,400 and 5,800 Å with a flat maximum between 6,000 and 6,200 Å. It will be noted that, while the density for equivalent amounts of galacturonic acid and its polymers is not identical, the absorption curves of all 3 compounds show an almost perfect parallelism between 5,400 and 6,000 Å.

(b) *The Absorption Increment between Two Wave Lengths as a Test for Galacturonic Acid.* To express the characteristic differences in absorption curves of galacturonic acid on one hand, and of the other hexuronic acids on the other, it seems most practical to calculate the difference between the extinction coefficient at 5,400 Å ( $E_{5,400}$ ) and 4,800 Å ( $E_{4,800}$ ).  $E_{5,400} - E_{4,800}$  is zero or slightly negative for glucuronic acid, polyglucuronides and alginic acid, and strongly positive for galacturonic and pectic acids and *Pneumococcus* polysacch. T.I (Table I). Any positive value of  $E_{5,400} - E_{4,800}$  in a solution of hexuronic acids will, therefore, indicate presence of galacturonic acid even if the other acids are in considerable excess.

TABLE I

*Extinction Coefficients (E) at Various Wave Lengths for the Cysteine Reaction of Hexuronic Acids and Polyuronides Determined with the Beckman Spectrophotometer*

Substance	Concentration in mg.-% of hexuronic acid	$E_{5,400}$	$E_{4,800}$	$E_{6,000}$	$E_{6,000} - E_{5,400}$
Glucuronic acid	160	0.065	0.045	0.060	-0.005
Hyaluronic acid	135	0.120	-0.115	0.110	-0.010
Chondroitinsulphuric acid from cartilage	150	0.360	0.375	0.330	-0.030
Galacturonic acid	80	0.440	0.530	0.665	0.225
Pectic acid	80	0.075		0.300	0.225
<i>Pneumococcus</i> polysaccharide T.I	80	0.675		1.015	0.340

<sup>3</sup> The content in hexuronic acids of the preparations of galacturonic, hyaluronic and chondroitinsulphuric acids was determined by the Freudenberg modification of the Lefèvre-Tollens method. That of *Pneumococcus* polysaccharides by titration.



The same procedure can also be carried out with the Klett photoelectric colorimeter. In this case the difference of densities with filters 62 and 54 is measured.  $D_{62} - D_{54}$  is again positive with galacturonic acid and the two corresponding polyuronides, and zero or negative with the other hexuronic compounds (see Table II).

TABLE II

*Cysteine Reaction of Hexuronic Acids and Polyuronides*  
(Klett photoelectric colorimeter readings for densities  
using various filters after 24 and 48 hours)

Substance	Concentration in mg.-% hexuronic acid	$D_{54}$		$D_{56}$		$D_{62}$		$D_{62}$	$D_{54}$
		24 hr.	48 hr.	24 hr.	48 hr.	24 hr.	48 hr.		
1. Glucuronic acid	160	41	68	30	581	47	82	6	14
2. Hyaluronic acid	135	43	75	58	85	56	85	13	10
3. Chondroitinsulphuric acid	150	179	232	206	280	88	120	-91	-112
4. Galacturonic acid	80	184	164	302	288	321	338	137	174
5. Pectic acid	80	20	45	113	186	197	226	177	181
6. <i>Pneumococcus</i> polysaccharide T.I	80	278	303	461	512	472	574	194	271
7. Alginic acid	80	3	0	25	16	27	24	24	24
8. Ribosenucleic acid	12	-2	-4			0	0	-2	-4
9. Galacturonic acid + ribosenucleic acid	80 12	169	140	272	204	287	310	118	170
10. Glucose	10	1	3	6		8	6	1	3
11. Alginic acid	200	70		95		55		-15	

(c) *Sensitivity of the Test.* As the reaction is positive with 0.04% of galacturonic acid in solution, it is possible to detect 0.2 mg. of galacturonic acid in free or combined form. When galacturonic acid must be detected in an excess of other hexuronic acids the total content in uronic acids of the solution should be 0.2%. At this concentration the other hexuronic acids do not give the reaction and it will be possible to detect galacturonic acid if its amount is more than 20% of the total hexuronic acids in solution. Whether concentrations above 0.2% of glucuronic and mannuronic acids give a negative test has not yet been investigated.

(d) *Influence of True Sugars on the Cysteine Reaction of Galacturonic Acid.* It was mentioned above that hexoses as well as pentoses react intensely with cysteine. The yellow color of hexoses has so low an absorption beyond the blue region of the spectrum, that, in general, their presence in purified preparations of polyuronides will not change the character of the absorption curve of the cysteine reaction in the region beyond 5,400 Å. Pentoses, on the other hand, show a sharp maximum of absorption at 5,400 Å which must distort this part of the curves of the hexuronic acids. Presence of pentoses, however, can easily be recognized by the fact that their absorption curves

show a steep rise between 4,400 and 5,400 Å and must, therefore, change completely the character of the curve of hexuronic acids in this region.

### DISCUSSION

The main advantage of the color reaction described in this report (apart from its specificity) seems to reside in the fact that the hexuronic acids in polyuronides react in the same way as free acids under conditions at which we cannot expect any considerable hydrolysis of the glucosidic linkages which bind the hexuronic constituents in the polyuronide. This becomes clear when the following facts are considered. If, in carrying out the cysteine reaction with galacturonic acid, the reaction mixture is cooled with ice water, instead of water at room temperature, the blue-green color does not appear. Only after the reaction mixture has been held at 70°C. for at least 90 seconds is the characteristic color produced. This shows that the color reaction of galacturonic acid depends on a decomposition product of the acid, which is formed during the few seconds in which the mixture of H<sub>2</sub>O and conc. H<sub>2</sub>SO<sub>4</sub> immersed in a water bath of 22-25°C. has a temperature of more than 70°C. Under these conditions, no significant amounts of free galacturonic acid are formed, as could be shown in the following way.

When 1 cc. of 1% solution of galacturonic acid is mixed, while cooling in water of 22°C., with 4 cc. of concentrated H<sub>2</sub>SO<sub>4</sub>, and, after a few minutes, the mixture is poured into 1.5 N NaOH equivalent to the 4 cc. of conc. H<sub>2</sub>SO<sub>4</sub>, the resulting solution shows no reducing power against K<sub>3</sub>Fe(CN)<sub>6</sub>, and the carbazole reaction of hexuronic acids is decreased by 83%. Galacturonic acid in the solution was, therefore, almost completely destroyed by this treatment. If, however, the same experiment is carried out with a 1% solution of *Pneumococcus* polysaccharide T.I., the carbazole reaction does not decrease by more than 8% and the originally non-reducing solution shows a reducing power corresponding to 25-30% of the total sugars. The solution no longer precipitates the specific horse antiserum in a dilution of 1:10,000. This shows clearly that the polysaccharide undergoes profound depolymerization on treatment with H<sub>2</sub>SO<sub>4</sub>, but the main bulk of galacturonic acid remains in combined form. Nevertheless, the cysteine reaction of the polysaccharide is not only not weaker than that of an equivalent amount of free galacturonic acid, but considerably stronger (Fig. 1). The specific intermediary product responsible for the cysteine

reaction of the polysaccharide must, therefore, be formed from the hexuronic acid combined by glucosidic linkages without prior liberation.

On the basis of these facts we may assume that this color reaction yields information on the state of hexuronic constituents in the molecule of polyuronide prior to any rearrangement which may occur during hydrolysis. Too little attention has been paid to the latter possibility in using methods involving treatment with acids for the identification of sugars in polysaccharides. For example, sugar anhydrides with an ethylene oxide ring were shown (2, 3) to undergo inversions on both carbon atoms connected by the secondary ring, when treated with diluted acid. The presence of such anhydrides in a polysaccharide may be overlooked and lead to erroneous identifications. A case in which such a possibility must be seriously considered will be discussed later. On the other hand, preliminary identifications based on color reaction must be considered with caution as long as the nature and mechanism of the color reaction are unknown. In this case, it is impossible to predict whether other compounds may not give the reaction. Apart from 3 hexuronic acids found in nature, others may occur and be found in the future. Conclusions can be drawn, however, in case our color reaction is negative in sufficiently concentrated solution. This indicates absence of galacturonic acid.

### SUMMARY

A new specific color reaction of galacturonic acid is described. It can be used to detect galacturonic acid in polyuronides in presence of an excess of other hexuronic acids.

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# Effect of Fat and Calories on the Resistance of Mice to 2,4-Dinitrotoluene<sup>1</sup>

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Received October 6, 1947

## INTRODUCTION

It has been shown that mice are more resistant to 2,4-dinitrotoluene (DNT) fed in a diet containing 5 or 30% of fat than in a low fat diet (1). This was evident both by the better growth and better survival of the animals. It was also noted that the consumption of food was greatest on the diets containing fat and, hence, mice on these diets were ingesting more calories and more DNT than those on the low-fat diet. The present experiments were designed to determine the relative effects of fat and of calories upon DNT toxicity. The diets used were compounded isocalorically, and controlled amounts of food were given.

## METHODS

Uniform groups of 3-6 weanling albino or C<sub>3</sub>H mice weighing 8-11 g. were placed in wire bottom cages and fed the diets listed in Table I. The food was weighed daily; water was given *ad libitum*. The mice were weighed individually twice a week and the amounts of food given, or consumed *ad libitum*, were calculated as calories/10 g. mouse daily. All significant experiments were performed at least twice.

The yeast diets I, II, and III (Table I) were compounded so that, for each calorie consumed, the amounts of DNT, casein, salt, and yeast would be constant as the amounts of fat and carbohydrate were varied. The high fat diet III contained 30% of added fat by weight and the medium fat diet II contained 5%. In addition, all diets, including the low fat diet, contained fat from the casein, yeast, and halibut liver oil; 0.46% of the low fat diet was extractable by ether. In the purified diets IV, V, and VI, cerelese was substituted for dextrin and synthetic vitamins were used instead of

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<sup>1</sup> Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation and the Jonathan Bowman Fund of the Medical School.

TABLE I  
*Composition of Diets<sup>a</sup>*

	I	II	III	IV <sup>b</sup>	V <sup>b</sup>	VI
Casein	18	18	18	18	18	18
Salts	4	4	4	4	4	4
Yeast	8	8	8	—	—	—
Dextrin	75	65	24	—	—	—
Cerelose	—	—	—	75.5	63.4	22.7
Corn oil	—	5	—	0.5	0.5	0.5
Hydro. cottonseed oil	—	—	23	—	4.1	19.5
Cellulflour	—	—	—	2	2	2
Total	105	100	77	100	92	66.7
±DNT	0.3	0.3	0.3	0.3	0.3	0.3
Per cent DNT	.285	.30	.39	.30	.33	.42
Per cent added fat	—	5	30	0.5	5.0	30
Cal./g.	3.89	4.14	5.35	3.35	3.62	4.98

<sup>a</sup> Halibut liver oil was added to all the rations or each mouse was given adequate amounts by dropper.

<sup>b</sup> The following levels (mg.) of synthetic vitamins were added/kg. equivalent of diets IV, V and VI:

Thiamine—10, Riboflavin—10, Pyridoxine—10,  
Calcium pantothenate—100, Nicotinic acid—100,  
Choline—300, Inositol—300, *p*-Aminobenzoic acid—200.

yeast. In the yeast series the percentage of DNT in the medium fat diet was 0.3% and the other diets contained equivalent amounts on a caloric basis. DNT at a level of 0.3% was incorporated into the low fat diet IV and diets V and VI contained equivalent amounts of DNT/cal. Diets IV and VI, therefore, contained somewhat more DNT than the corresponding diets I to III.

## EXPERIMENTAL AND RESULTS

Typical results are indicated in Table II. On both the yeast diets and the synthetic diets, growth in the presence of DNT was best on the high fat diet and poorest on the diet low in fat, while the variations in the growth of mice not fed DNT were relatively minor. The mice fed the high fat diets plus DNT grew best in spite of the fact that the percentage of DNT in the diet was 0.39% as contrasted to only 0.285% in the low-fat diet. Mice fed control diets free from DNT tended to consume approximately the same number of calories daily throughout the

TABLE II

*Growth and Food Consumption ad libitum of Mice Fed Isocaloric Diets Containing 2,4-Dinitrotoluene (DNT)<sup>a</sup>*

Diet	Cal./mouse/day			Cal./10 g. mouse/day			Av. gain per week for 3 weeks
	1st wk.	2nd wk.	3d wk.	1st wk.	2nd wk.	3d wk.	
I-Low fat-DNT	11.4	12.8	12.5	8.0	7.4	6.2	3.9
I-Low fat+DNT	8.9	6.5	7.3	7.3	4.6	3.7	(1.6 <sup>a</sup> )
II-Med. fat-DNT	10.0	14.1	11.5	10.5	9.0	5.6	4.4
II-Med. fat+DNT	6.6	8.0	7.3	6.4	6.7	5.2	1.5
III-High fat-DNT	12.0	13.7	13.5	9.0	7.3	6.0	4.2
III-High fat+DNT	9.5	10.2	9.0	7.8	6.9	5.5	2.0
IV-Low fat-DNT	9.1	9.1	9.7	6.7	5.7	5.3	2.1
IV-Low fat+DNT	8.1	6.9	6.7	6.1	5.1	4.7	.4
V-Med. fat-DNT	10.9	10.3	9.3	7.4	6.0	5.2	1.0
V-Med. fat+DNT	9.9	9.3	9.5	6.8	5.9	5.4	1.4
VI-High fat-DNT	13.2	14.6	14.2	8.1	7.4	6.2	3.1
VI-High fat+DNT	10.8	13.0	12.0	7.8	8.0	6.6	1.6

<sup>a</sup> Five mice per group. All mice in this series survived for 5 weeks except one in the group fed DNT in the low fat diet I, which died during the third week.

experimental period of 3 weeks, and since the animals were gaining in weight, the consumption calculated as cal./10 g. mouse daily decreased as the experiment continued. The number of calories consumed was somewhat higher on the high fat diet than on that low in fat, and this difference was aggravated in the presence of DNT.

A survey of the results of 5 different series indicated that mice not fed the nitro compound usually chose to eat more calories when the diet contained 30% of fat than when the percentage of fat was only 0.46% (Table III). The average increase in caloric intake on the high fat diet as compared with the low was 13.1%. On the medium fat diet, caloric intake was increased by only 7.5%. These differences were greatly accentuated when DNT was present in the diet. Mice fed the medium fat diet plus DNT consumed 15.7% more calories than those on the low fat diet plus DNT; on the high fat diet the caloric intake was 32.4% greater than on the corresponding diet low in fat. These differences became even greater as exposure to the nitro compound continued (Table II). In this connection it is of interest to note that mice painted with methylcholanthrene and fed a high fat diet consumed 12-30%

TABLE III

*Voluntary Caloric Intake of Mice Fed Different Percentages of Dietary Fat With or Without 2,4-Dinitrotoluene (DNT)*

Weeks on diet	No. mice per group	Calories/10 g. mouse/day			Per cent increase over low fat diet	
		Low fat	Med. fat	High fat	Med. fat	High fat
-DNT						
2	3	8.07	—	9.22	—	14.2
2	4	5.72	6.35	6.65	11.0	16.6
2	3	6.6	6.7	7.4	1.5	12.0
3	4	7.5	8.3	7.4	10.7	-1.3
3	5	5.8	6.2	7.2	6.9	24.2
Average	—	—	—	—	7.5	13.1
+DNT						
2	4	5.72	6.47	7.37	13.1	28.6
1	4	5.2	6.4	6.9	19.0	32.7
3	4	5.2	6.1	6.7	17.3	28.8
3	5	5.3	6.0	7.4	13.2	39.6
Average	—	—	—	—	15.7	32.4

more calories than similarly treated animals on a low-fat diet (2). The results suggest that an important reason for the poor performance of mice on low-fat diets plus DNT is that such animals tend to stop eating. Mice on the high fat diets, on the other hand, grew fairly well even though their intake of DNT (and calories) was greater than that of mice on the low fat diet. Apparently, therefore, the extra calories more than compensated for the harmful effects of the additional DNT consumed by the mice fed the high fat diet.

The great importance of calories on the growth of mice fed DNT was indicated in another experiment in which diets I and III were fed to mice in amounts that supplied roughly the same number of calories and DNT/10 g. mouse for 2 weeks, while control animals received similar amounts of the high and low fat diets without the nitro compound.

As in previous series, the mice receiving DNT grew somewhat less than those not exposed to the compound, while those getting DNT in a high fat diet grew better than those on the low fat diet. After the first 2 weeks, both groups were fed equal amounts of DNT, amounts consumed voluntarily by the mice on the low fat diet, but the animals on the high fat diet were allowed to consume as much additional control

ration III as they wished. The amounts of DNT consumed were 2.6 and 1.9 mg./10 g. mouse/day during the third and fourth weeks, respectively; in the same period the caloric intake averaged 4.0 cal./10 g. mouse/day for the low fat group and 7.1 for those on the high fat diet. Under these conditions the mice on the high fat diet plus DNT gained 4.6 g. in 2 weeks, as much as those not receiving the compound, while the mice on the low fat diet lost 1.0 g., and one of 7 mice died. An unexpected observation was that the control mice on the low fat diet lost more weight (1.7 g.) and died sooner (5 of 7 dead) than those receiving a similar amount of food containing the nitro compounds (loss in 2 weeks, 1.0 g.; 1 of 7 dead), although the latter animals were in poor physical condition, with rough coats, inactive, and cold to the touch. Apparently, the better survival of the poisoned animals was due to their lack of activity, since similarly starved controls ran about the cage searching for food, thus depleting their body reserves more rapidly.

To distinguish between the specific effects of DNT and those of self-imposed starvation, mice were fed different percentages of fat but the same number of calories; diets I, II and III with or without DNT in amounts to supply 5, 5.7, 6 or 7 cal./day/10 g. mouse. At the 5 cal. level mice fed DNT maintained weight as well as those not fed the compound, except those on the low fat diet which lost an average of 0.8 g./week (Table IV). Again, survival on the low fat diet was slightly better

TABLE IV  
*Relative Effect of Calories, Fat, and 2,4-Dinitrotoluene (DNT) on  
the Growth and Survival of Young Mice*

Diet	Cal./10 g. mouse/day	Av. initial weight	Gain per week <sup>a</sup>		Survival	
			-DNT	+DNT	-DNT	+DNT
I-Low fat	5	9.8	0	-0.8	3/4	4/4
II-Med. fat	5	10.2	-0.45	-0.45	3/4	4/4
III-High fat	5	10.4	-0.15	-0.15	3/4	4/4
I-Low fat	5.7	9.2	1.2	-2.6 <sup>b</sup>	3/4	3/4
II-Med. fat	5.7	8.6	1.9	0.7 <sup>b</sup>	4/4	4/4
III-High fat	5.7	9.2	0.5	1.3	3/4	3/4
I-Low fat	6	11.5	0.9	-1.7 <sup>b</sup>	10/10	10/10
II-Med. fat	6	9.5	1.0	0	10/10	10/10
III-High fat	6	9.6	0.7	0.7	7/10	9/10
I-Low fat	7	9.8	1.9		4/4	
II-Med. fat	7	11.0	1.8		4/4	
III-High fat	7	10.5	1.9		4/4	

<sup>a</sup> Weekly average over a 2 week period.

<sup>b</sup> These animals did not eat their full allotment of food and so did not receive the entire amount of calories indicated.



when DNT was present in the diet than when it was absent. At the 5.7 and 6 cal. levels, mice on the low fat diet (Diet I) plus DNT did not consume all of their food, but at the other levels of fat the rate of growth increased as the level of fat in the diet increased in the presence of DNT, indicating some protection due to fat *per se*. The animals receiving the control diets free from DNT grew as well or better than corresponding mice fed the compound and the growth on all groups of the control diets were nearly equal. At the 7 cal. level the food was not all consumed when DNT was present but in the absence of DNT the growth on the 3 levels of fat was equal for a 2 week period.

Apparently most natural fats confer an increased resistance to DNT. The fats fed included cottonseed oil, corn oil, peanut oil, partially hydrogenated cottonseed oil (Crisco), hydrogenated cocoanut oil, lard, and butter fat at a level of 15% in a ration based on diet I (Table I). The level of DNT in these high fat diets was 0.33%. No consistent differences among these fats were evident, and growth was nearly always better than on an isocaloric diet low in fat. The poorest growth in the presence of DNT was observed on a rancid sample of partially hydrogenated vegetable oil with a peroxide number of 19.3 as compared to 0 for the fresh material. In the absence of DNT, however, growth on this rancid fat was essentially the same as when the fresh fat was fed.

In parallel experiments rats were found to resemble mice in their general response to fat, calories, and dinitrotoluene. With isocaloric amounts of food and DNT (diets I, II, and III, Table I) so that consumption/g. of rat was equal, growth in 6 weeks on the low, medium, and high fat diets was 21, 30, and 48 g., respectively. In other experiments it was observed that rats on the low fat diet tended to consume the least calories, and that with *ad libitum* feeding, growth in the presence of DNT was improved as the level of fat in the diet increased.

## DISCUSSION

Apparently, the growth and survival of mice (and rats) exposed to 2,4-dinitrotoluene is determined to a large extent by their caloric intake. Mice receiving an adequate number of calories can consume fairly large amounts of the nitro compound without obvious damage to the animal in a short term experiment. On the other hand, mice fed DNT in low fat diets do very poorly, largely because they stop eating, although mice fed similar amounts of DNT in the presence of fat continue to eat normally. The presence of fat in diets containing DNT might conceivably improve their taste, but a metabolic factor is sug-

gested by the observation that mice fed DNT grew best on high fat diets even though the intake of calories was equalized. Fat probably brings about a more efficient metabolism than that found in mice not fed fat. Forbes and Swift (3) have demonstrated that the specific dynamic action of a food stuff depends upon the proportion of fat, protein, and carbohydrate in the diet, and that protein, for example, is utilized most efficiently in the presence of fat. It is, therefore, possible that mice receiving fat are better able to convert DNT into harmless derivatives than mice fed low-fat diets.

White, Foy and Cerecedo (4) have observed suboptimal growth as well as the typical Burr and Burr syndrome in mice fed a diet rigidly devoid of fat. When 10% of lard was added to their diets, the mice gained 2.3 g./week during the first 3 weeks of the experiment. In a similar period, our mice gained from 2.4 to 3.3 g./week on the so-called "low fat diet" without developing any symptoms of deficiency, and hence the diet must have supplied enough essential fatty acids for general well-being. Apparently, the effects of fat reported in the present paper represent those above the minimum needed in the absence of an abnormal physiological strain such as the DNT imposes.

The present study also has a bearing on a limitation of the paired feeding technique. Under the proper experimental conditions mice on the low fat diet fed DNT lost less weight and lived longer than control mice not fed DNT but restricted to a similar intake of food. By the process of reasoning used in connection with paired feeding experiments, it might, therefore, be argued that dinitrotoluene is a growth factor! However, the true explanation of the difference in the growth and survival of these two groups seems to lie in the greater activity of the control mice not fed DNT, with a more rapid depletion of body reserves than by the more sluggish mice poisoned with DNT. In other words, the logic behind a paired feeding experiment presupposes that the physical activity of the two groups being compared is equal.

#### SUMMARY

1. Mice ingesting 2,4-dinitrotoluene (DNT) grew less and died faster when fed a diet moderately low in fat (0.46%) than when fed the same amount of nitro compound per calorie in diets containing 5 or 30% of added fat. Fat likewise appeared to minimize the effects of DNT in rats.

2. Many of the harmful effects observed on the low fat diet appeared to be due to the low caloric intake of the animals. Mice fed the low fat diet plus DNT grew for only 2 weeks and then lost weight. Their in-

take of food became progressively less. After 2 weeks it was so low that similarly restricted mice not receiving DNT also lost weight and died. In the absence of DNT, mice grew well on this low fat diet fed *ad libitum*.

3. On all 3 levels of fat fed, DNT *per se* retarded growth only slightly during the early stages of the experiment when the mice consumed 6-7 cal./10 g. mouse daily. When less than 5 cal. were fed per day, the control animals, being more active, sometimes lost more weight and died sooner than those receiving the nitro compound.

4. The following fats appeared to be equally active in minimizing the effects of DNT in mice: cottonseed oil, corn oil, peanut oil, partially hydrogenated cottonseed oil (Crisco), hydrogenated coconut oil, lard, and butter fat. Rancid hydrogenated cottonseed oil increased the effects of the nitro compound.

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# Antibacterial Unsaturated Ketones and Their Mode of Action<sup>1,2</sup>

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Received October 13, 1947

## INTRODUCTION

Acrylophenone and two of its derivatives, benzalacetophenone and furfuralacetophenone, were previously reported to have antibacterial and antifungal properties (1). Acrylophenone was considerably more potent than the others, as it prevented the growth of *Staphylococcus aureus* at 1:200,000, of *Escherichia coli* at 1:30,000, and of the *Trichophyton mentagrophytes* at 1:3,000,000. It was suggested that the antibacterial properties of these substances were a result of their ability to react with sulfhydryl groups. Amstutz, Fehnel and Neumoyer (2) have also reported that derivatives of  $\alpha$ -methylacrylophenone are active against *S. aureus*. The antibacterial and antifungal properties of a number of other  $\alpha,\beta$ -unsaturated ketones have been studied, and further information has been obtained concerning their mode of action.

Four classes of unsaturated ketones have been included in the present study: benzoylpyruvic acid and a derivative, benzoylacrylic acid and two derivatives, derivatives of acrylophenone with substitution in the benzene ring, and dibenzoylethylene and its derivatives.

## EXPERIMENTAL

### *Materials*

The sources of the unsaturated ketones and  $\beta$ -chloroketones used in the present study are indicated in a subsequent table (Refs. 3-18). Modifications and improvements were made in the published procedures in some instances (14). The chloro-

<sup>1</sup>Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.

<sup>2</sup>These investigations were supported in part by a grant from The Rohm and Haas Co.

ketones (10-14) were converted to the corresponding acrylophenone derivatives by an extension of the method of Allen *et al.* (3), who observed that  $\beta$ -chloropropiophenone was decomposed on warming with alcoholic potassium acetate to yield acrylophenone. Many of the unsaturated ketones were, therefore, not isolated, but solutions were prepared as needed from the corresponding  $\beta$ -chloropropionyl ketones for bacteriological testing. Although it was found that the  $\beta$ -chloropropionyl ketones were as active against bacteria and fungi as the unsaturated ketones derived from them, this seemed to be explained by the fact that the  $\beta$ -chloropropionyl derivatives were decomposed by nutrient agar with the liberation of acid and chloride. Nevertheless, the treatment with potassium acetate was regularly used as a precaution against extraneous effects due to the formation of hydrochloric acid.

*S-( $\beta$ -2-Hydroxy-5-methylbenzoyl)-ethylthioglycolic Acid*

2-Hydroxy-5-methyl- $\beta$ -chloropropiophenone (363 mg.) was refluxed for 10 minutes with 10 ml. of ethyl alcohol and 200 mg. of potassium acetate. Sodium thioglycolate solution was added in excess, and the solution acidified, diluted with water, and chilled. An oil, which slowly crystallized, separated. The supernatant was decanted, and the residue dried *in vacuo* and then crystallized from a mixture of chloroform and ligroin. The product consisted of hygroscopic white crystals weighing 224 mg., and melting at 70°C. *Anal.* Calcd. for  $C_{12}H_{14}O_4S$ : C, 56.69; H, 5.52; S, 12.60. Found: C, 56.45; H, 5.86; S, 13.29.

The microanalysis of this and the subsequent compound was carried out by Joseph F. Alicino. A bright purple color, on adding dilute ferric chloride to a solution of the compound, confirmed the presence of a phenolic group.

*S-(1,2-Dibenzoyl-ethyl)-thioglycolic Acid*

*Trans*-dibenzoyl-ethylene (226 mg.) was dissolved in 10 ml. of hot alcohol, and sodium thioglycolate solution was added until the yellow color of the ketone had disappeared. The mixture was acidified, diluted with water, and the product filtered off. After one crystallization, 302 mg. of a white crystalline product melting at 163°C. were obtained. *Anal.* Calcd. for  $C_{18}H_{16}O_4S$ : C, 67.77; H, 5.03; S, 10.06. Found: C, 65.29; H, 6.98; S, 9.91. The deviations from theory are attributed to the easy dissociation of the compound into its components.

*Antibacterial and Antifungal Tests*

The test methods used in the present work were essentially the same as in previous papers (1, 20). Solutions of the compounds were tested by the agar-streak method (19). With the simpler derivatives of acrylophenone, the alcoholic solutions of the ketones were diluted with water. With the derivatives of dibenzoyl-ethylene, the dilutions were made with alcohol or acetone. No more than 0.3 ml. of alcohol or 0.5 ml. acetone was added to any plate containing 10 ml. of agar.

The organisms used for the test with acid-fast bacteria were a strain of *Mycobacterium phlei* isolated in this laboratory, and a so-called non-pathogenic fast-growing strain of *M. tuberculosis* (ATCC 607). In the opinion of some investigators this latter organism is actually an acid-fast saprophyte.

### Metabolic Studies

The preparation of succinoxidase was made according to Ball and Ormsbee (21), and was diluted with Krebs Ringer-phosphate solution (22). The cytochrome c was prepared according to Keilin and Hartree (23), and the presence of optimum levels of this component was assured by preliminary experiments. The methods used in studying the action of the ketones upon the succinoxidase system and upon succinic dehydrogenase follow those used by Ball, Anfinsen and Cooper (24) in studying the action of naphthoquinones upon this same enzyme. The ketones were usually dissolved in ethanol, but, in the experiments with yeast, solutions in methanol were used. The organisms used included a previously used strain of *E. coli* (1, 20), and *Micrococcus lysodeikticus* (ATCC 4698). The cell suspensions of *E. coli* were prepared as previously described (25). The cell suspension of *M. lysodeikticus* was prepared by growing the organism on nutrient agar for 48 hours at 2°C., and washing the cells off the surface with 0.9% sodium chloride. The cells were then washed 3 times further with this salt solution, and finally were suspended in this same solution. The nitrogen content of the cell suspensions was determined by the micro Kjeldahl procedure of Clarke (26).

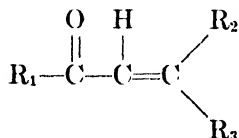
Lysis of *M. lysodeikticus* was accomplished by mixing 1.0 ml. of cell suspension (1.4 mg. bacterial N/ml.) with 1.0 ml. of filtered saliva and 8.0 ml. of water. A similar procedure has been used by Krampitz and Werkman (27). Manometric readings were begun one hour after mixing. Because the organism was found to contain an active alcohol-oxidizing system, which fortunately was not affected by the ketones, control experiments were run to determine what part of the oxygen uptake was due to oxidation of the alcohol used as a solvent for the ketones.

The acetone-treated yeast was prepared by first washing 20 g. of Anheuser-Busch bakers' yeast with three 200 ml. portions of water. The yeast was then shaken with 200 ml. of acetone, centrifuged, and dried under a fan. Results obtained by Meyerhof (28) indicate that acetone-treated yeast contains triose phosphate dehydrogenase. Sodium hexose diphosphate, prepared from the commercial barium salt, was used as a source of triose phosphate.

## RESULTS AND DISCUSSION

### *Effect of Structure upon Activity*

Two rules were derived from earlier work (1) which relate the structure of an  $\alpha,\beta$ -unsaturated ketone to its antibacterial activity. In a ketone of the general structure



antibacterial activity was favored when: (a)  $\text{R}_1$  was an aryl group;

TABLE I  
Antibacterial and Antifungal Properties of  $\alpha,\beta$ -Unsaturated Ketones

	Dilution units/mg. (17) against								Reference and source
	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>	<i>Bacillus subtilis</i>	<i>Aspergillus niger</i>	<i>Trichoderma koningi</i>	<i>Cryptococcus neoformans</i>	<i>Trichophyton mentagrophytes</i>	
Benzalpyruvic acid	<3	<3	3	3	<3	<3	<3	3	(4) F
Anisalpyruvic acid	<3	2	10	20	<3	<3	<3	5	(5) F
Benzoylacrylic acid	10	3	20	50	<3	<3	<3	5	(6) F,L
p-Bromobenzoylacrylic acid	<3	<3	200	200	3	3	3	3	(7) L
β-Mesitoylacrylic acid	<3	<3	1,000	100	3	<3	3	<3	(8) L
4-Aminobenzalacetophenone	<30	<30	30	30	100	200	100	300	(9) L
Methyl vinyl ketone	10	10	10	10	10	100	100	200	D
AP <sup>a</sup>	30	3	200	200	20	100	30	1,000	(3)
4-Methyl-AP	10	5	300	1,000	3	100	300	3,000	(10,11)F
4-Methoxy-AP	8	3	30	80	3	30	30	>300	(10,11)F
4-Chloro-AP	10	5	500	500	20	100	100	3,000	(10) F
2-Hydroxy-AP	100	10	100	300	100	300	100	300	(12) F
2-Hydroxy-5-chloro-AP	10	10	100	100	10	10	300	300	(14) F
2-Hydroxy-5-methyl-AP	10	3	1,000	1,000	80	300	>1,000	>1,000	(13) F
2-Hydroxy-4-methyl-AP	10	4	500	500	50	100	500	500	(14) F
4-Hydroxy-2-methyl-AP	10	3	30	100	3	400	1,000	1,000	(14) F
2-Hydroxy-3-methyl-AP	3	3	50	50	10	20	100	200	(14) F

<sup>a</sup> AP = acrylophenone.

<sup>b</sup> E = ethylene.

<sup>c</sup> F indicates preparation of the compound or its HCl-adduct by M. C. Fernald; L, by R. E. Lutz; D, by E. I. du Pont de Nemours and Co., Inc.

TABLE I (continued)

	Dilution units/mg. (17) against							Reference and source	
	<i>Esche- richia coli</i>	<i>Pseudo- monas aeruginosa</i>	<i>Staphylo- coccus aureus</i>	<i>Bacillus subtilis</i>	<i>Aspergil- lus niger</i>	<i>Tricho- derma koningii</i>	<i>Cryptococ- cus neo- formans</i>		<i>Trichophy- ton menia- grophyles</i>
4-Hydroxy-3-methyl-AP	3	2	50	100	10	10	50	500	(14) F
2-Hydroxy-4,6-dimethyl-AP	3	3	300	300	30	100	200	1,000	(14) F
2-Hydroxy-5-methoxy-AP	5	3	100	200	5	30	20	300	(14) F
2-Methyl-4-hydroxy-5- isopropyl-AP	2	2	20	30	50	50		300	(13) F
trans-Dibenzoyl-E <sup>b</sup>	40	20	1,000	1,000	300	300	200	3,000	(15) F
cis-Dibenzoyl-E	100	20	3,000	3,000	300	1,000	1,000	1,000	(15) L
Dibenzoyl ethane	<3	<3	<3	<3	<3	<3	<3	<3	(15)
Dibenzoyl acetylene	<3	<3	<3	<3	<3	<3	<3	<3	(16) L
cis-Dibenzoyldichloro-E	<30	<30	2,000	3,000	300	300	1,000	3,000	(17) L
trans-Dibenzoyldimethyl-E-	<3	<3	<3	<3	<3	<3	<3	<3	(18) L
1-Benzoyl-2-mesitoyl-E	20	20	3,000	3,000	100	1,000	>3,000	>3,000	L
trans-Dimesitoyl-E	<30	<30	<30	<30	<30	<30	<30	<30	(15) L
trans-Dimesitoyldimethyl-E	<30	<30	<30	<30	<30	<30	<30	<30	(18) L
trans-Di-(4-isopropyl benzoyl)-E	<30	<30	2,000	1,000	<30	<30	50	50	L
Di-4-methylbenzoyl-E	<20	<20	5,000	3,000	20	<20	<20	5,000	(17) F
Di-4-chlorobenzoyl-E	<3	<3	3,000	3,000	300	300	<20	300	(17) F
Di-(5-methyl-2- chlorobenzoyl)-E	<3	<3	3,000	3,000	300	300	20	2,000	(17) F
Di-(3-methyl-4- chlorobenzoyl)-E	<3	<3	100	300	300	20	<20	700	(17) F



(b)  $R_2$  or  $R_3$  (or both) was hydrogen. The results of Amstutz, Fehnel and Neumoyer (2), who studied derivatives of  $\alpha$ -methylacrylophenone, tend to confirm the importance of a lack of substituents in the sidechain. The first 7 compounds listed in Table I are some that do not conform to these rules. All were found to have rather low activity, particularly against gram-negative bacteria such as *E. coli* and *Pseudomonas aeruginosa*, and against fungi. Benzalpyruvic acid is of interest because it has been reported to react rapidly and spontaneously with mercaptans (29). In these 7 compounds, it appears that substitution in the sidechain or elimination of the phenyl group leads to low activity.

For this reason attention was directed to the effect of substitution in the aromatic nucleus upon the activity of acrylophenone derivatives. The introduction of a methyl group or chlorine atom was found to be of little advantage, while the introduction of a methoxyl group led to low solubility and decreased activity. The compounds that contained a phenolic hydroxyl group were, in contrast, found to be much more soluble, and some of them were much more active than acrylophenone. The presence of a hydroxyl group in a position *ortho* to the ketonic group, as in 2-hydroxyacrylophenone, 2-hydroxy-4-methylacrylophenone, and 2-hydroxy-5-methylacrylophenone, seemed to be especially favorable to high activity. These compounds were noteworthy for their activity against the gram-negative *E. coli*, and against all the gram-positive bacteria, since they inhibited the former at dilutions as high as 1:200,000, and the latter at dilutions of 1:1,000,000. The 2-hydroxy compounds were more active than their 4-hydroxy isomers.

Both *cis*- and *trans*-dibenzoylethylene, and many derivatives with substituents in the phenyl groups, were found to be quite active. This was unexpected, since these compounds may be considered to be derivatives of acrylophenone with a substituent in the sidechain, and, there, fail to conform with rule (b). These compounds were active against gram-positive bacteria, such as *S. aureus* and *Bacillus subtilis*, at dilutions as high as 1:1,000,000 and against pathogenic fungi (*Cryptococcus* and *Trichophyton*) at dilutions of 1:3,000,000. Their activity against the gram-negative bacteria was only moderate, and the derivatives bearing a halogen atom or isopropyl group on the phenyl groups were so insoluble that concentrations sufficient to inhibit the gram-negative bacteria could not be attained. Dimesitylethylene was inactive, while benzoylmesitylethylene was about as active as di-

benzoylethylene. This may be due to steric factors (30). Dibenzoyl-dimethylethylene, dibenzoylacetylene, and dibenzoylethane were inactive. Several of the more active compounds were also tested against acid-fast bacteria (Table II). Dibenzoylethylene and some of its de-

TABLE II  
*Effect of Unsaturated Ketones on Acid-Fast Bacteria*

	Dilution units/mg. against <i>Mycobacterium tuberculosis</i>	Dilution units/mg. against <i>Mycobacterium phlei</i>
4-Chloro-AP	100	200
2-Hydroxy-5-methyl-AP	150	300
2-Hydroxy-4-methyl-AP	100	150
Dibenzoyl-E	2,000	2,000
Di-4-methylbenzoyl-E	2,000	2,000
Di-4-chlorobenzoyl-E	1,000	3,000
Di-(5-methyl-2-chlorobenzoyl)-E	1,000	4,000
Di-(3-methyl-4-chlorobenzoyl)-E	30	100

rivatives were active at dilutions greater than 1:1,000,000. Tests at Merck and Co. have shown dibenzoylethylene to be active against *M. tuberculosis* H37rv and MT-2 at 1:200,000 (31).

#### *Mode of Action*

Two compounds, *trans*-dibenzoylethylene and 2-hydroxy-5-methylacrylophenone, each of which seemed to be representative of a group of antibacterial unsaturated ketones, were selected for more detailed study. A preliminary investigation of the effect of sulfhydryl compounds on the ketones was carried out by methods used previously (1, 20). The results given in Table III show that thioglycolate and monothioglycol greatly diminish the activity of 2-hydroxy-5-methylacrylophenone against both gram-positive and gram-negative bacteria. This is typical of unsaturated ketones (1). Although solutions of dibenzoylethylene were observed to be decolorized by the sulfhydryl compounds, the reaction mixture was active against gram-positive but not against gram-negative bacteria (Table III). The compounds formed by the reaction of the two ketones with thioglycolic acid were isolated and tested, with results in agreement with those of the preliminary experiment. The compound formed from 2-hydroxy-5-methylacrylophenone and thioglycolic acid was found to lack anti-

TABLE III

*Effect of Sulphydryl Compounds on the Bacteriostatic Action of Unsaturated Ketones*

Substance	Sulphydryl compound	Dilution units/mg. of substance against			
		<i>Escherichia coli</i>	<i>Aerobacter aerogenes</i>	<i>Staphylococcus aureus</i>	<i>Bacillus subtilis</i>
2-Hydroxy-5-methyl-AP	None	10	10	1,000	1,000
	Thioglycolate	3	2	20	20
	Monothioglycol	2	2	20	50
Dibenzoyl-E	None	40	80	1,000	1,000
	Thioglycolate	2	2	1,000	2,000
	Monothioglycol	2	2	1,000	2,000
<i>S</i> -( $\beta$ -2-Hydroxy-5-methylbenzoyl)-ethylthioglycolic acid		<3	<3	1,000	200
<i>S</i> -(1,2-Dibenzoyl-ethyl)-thioglycolic acid		<3	<3	10	10

bacterial activity, while that formed from dibenzoyl-ethylene was active against gram-positive bacteria. When a solution of this latter compound in dilute aqueous sodium carbonate was warmed, the yellow color of dibenzoyl-ethylene reappeared, and the solution gave a positive nitroprusside test. The result suggests that dibenzoyl-ethylene forms a less stable linkage with sulphydryl compounds than does 2-hydroxy-5-methylacrylophenone.

Dibenzoyl-ethylene resembles quinones in this biological property by not being completely inactivated by mercaptans (20), and chemically in being an unsaturated 1,4-diketone. It therefore became of interest to investigate the action of the substance upon succinoxidase systems. One member of this system, succinic dehydrogenase, is an enzyme typical of those poisoned by reagents reacting with sulphydryl groups, while another member of the system located approximately at the cytochrome b level is inactivated by 2,3-disubstituted 1,4-naphthoquinones (24), which are not inactivated by mercaptans.

Investigation showed that the succinoxidase system of beef heart was completely inhibited by dibenzoyl-ethylene at levels as low as  $2.4 \times 10^{-4} M$  (Table IV). Experiments by the Thunberg technique

TABLE IV

*Effect of Unsaturated Ketones upon Succinoxidase*

To 2.0 ml of the succinoxidase preparation (0.0364 mg. N/ml.), *M. lysodeikticus* suspension (0.140 mg. bacterial N/ml.), or *E. coli* suspension (0.105 mg. bacterial N/ml.), was added 0.1 ml. of an alcoholic solution of the ketone. After equilibration at 38°C., sodium succinate (0.2 ml., 0.1 *M*) was tipped in and the oxygen uptake determined. In the fourth experiment, the flasks also contained cyanide (0.001 *M*), and methylene blue (0.2 ml., 0.1%) was added 10 minutes after adding the succinate (21).

Enzyme system	Oxygen uptake <sup>a</sup> at indicated concn. of inhibitor				None
	2.4 × 10 <sup>-3</sup> <i>M</i>	2.4 × 10 <sup>-4</sup> <i>M</i>	8.0 × 10 <sup>-5</sup> <i>M</i>	2.4 × 10 <sup>-5</sup> <i>M</i>	
Dibenzoyl-E as inhibitor:					
Succinoxidase <sup>b</sup>	0	0	1,200	1,850	2,120
<i>E. coli</i>	85	395	600	730	820
<i>M. lysodeikticus</i>	510	550	778	830	1,030
Succinic dehydrogenase <sup>b</sup>	0	0	190	760	800
2-Hydroxy-5-methyl-AP as inhibitor:					
Succinoxidase <sup>b</sup>	0	38	1,360	1,790	2,120
<i>E. coli</i>	33	430	590	740	820
<i>M. lysodeikticus</i>	270	600	770	810	870

<sup>a</sup>  $\mu\text{L O}_2/\text{mg. N} \times \text{hr.}$

<sup>b</sup> From beef heart.

(Table V) showed that succinoxidase was the component attacked. In these experiments it was noticed that no inhibition was observed if the dibenzoyl ethylene and succinate were added simultaneously, but that the dibenzoyl ethylene must be added first. Inhibition at the dehydrogenase level was confirmed by the method of Ball, Aufinsen and Cooper (24), in which the oxygen uptake in the presence of methylene blue and cyanide is measured (Table IV).

A study was next made of the effect of dibenzoyl ethylene upon the succinate oxidizing system of lysed cells of *M. lysodeikticus*, an organism whose growth is inhibited by dibenzoyl ethylene at high dilutions. Here it was found that dibenzoyl ethylene at a concentration of  $2.4 \times 10^{-4}$  *M* inhibited the oxygen uptake in the presence of succinate by about 50%, while increasing the concentration of the ketone to  $2.4 \times 10^{-3}$  *M* had little further effect. The methylene blue techniques were

found to be unsuited to studying the dehydrogenase system of this bacterium since the lysed cells reduced the dye rapidly in the absence of added substrate. It was noted that this endogenous reduction of the dye was inhibited by dibenzoyl ethylene.

The oxidation of succinate by *E. coli* was also found to be inhibited by dibenzoyl ethylene, but rather high levels,  $2.4 \times 10^{-3}$  M, were required. Studies by Thunberg technique (Table V) indicated that

TABLE V

*Effect of Unsaturated Ketones upon Dehydrogenases*

In the main compartment of Thunberg tubes were placed 2.0 ml. of the succinic dehydrogenase preparation or *E. coli* suspension (as in Table IV), or yeast suspension, at 25°C., and 0.1 ml. of a solution of the ketone. After evacuation, the succinate (0.2 ml., 0.1 M), alcohol (0.2 ml., 1.0 M), or hexose diphosphate (0.4 ml., 0.1 M), and the methylene blue (0.2 ml., 1:10,000) were tipped in.

Enzyme system	Reduction time at indicated concn. of inhibitor:					
	$2.4 \times 10^{-2}$ M	$8.0 \times 10^{-4}$ M	$2.4 \times 10^{-4}$ M	$8.0 \times 10^{-5}$ M	None	None <sup>a</sup>
Dibenzoyl-E as inhibitor:						
Succinate + dehydrogenase	> 60		60	20	12	> 60
Succinate + <i>E. coli</i>	9				4	26
Alcohol + yeast <sup>b</sup>	> 60		30	17	10	> 60
Hexose diphosphate + yeast <sup>c</sup>	45	10	7		5	25
2-Hydroxy-5-methyl-AP as inhibitor:						
Succinate + dehydrogenase	> 60		30	12	12	> 60
Succinate + <i>E. coli</i>	12				4	26
Alcohol + yeast <sup>b</sup>	> 60		12		10	> 60
Hexose diphosphate + yeast <sup>c</sup>	20	12	7		5	25

<sup>a</sup> Substrate omitted.

<sup>b</sup> With 10 mg. yeast per tube.

<sup>c</sup> With 20 mg. yeast per tube.

succinic dehydrogenase was the component reacting with the ketone.

Investigation of 2-hydroxy-5-methylacrylophenone led to rather similar conclusions as far as the succinoxidase of beef heart and of *E. coli* were concerned: roughly the same amount of inhibition was

brought about by the same concentrations of ketone. With *M. lysodeikticus*, a more complete inhibition of the oxidation of succinate was observed at higher concentrations of the ketone, instead of the leveling off at 50% inhibition observed with dibenzoyl ethylene.

Succinic dehydrogenase is inhibited not only by sulfhydryl reagents (32, 33, 34), but by malonate (35), a "metabolite analog" of succinate. Both types of compound seem to react at the same site (32). Therefore, it seemed possible that dibenzoyl ethylene might have a special affinity for succinic dehydrogenase, both because this ketone reacts with sulfhydryl groups, and because it is structurally related to fumarate, one of the substrates of this enzyme. Woolley (36) has noted that the replacement of carboxyl groups by phenylketo groups often leads to the production of inhibitory metabolite analogs. The action of the two ketones upon the alcohol dehydrogenase and triose phosphate dehydrogenase systems of yeast, which are known to be inhibited by sulfhydryl reagents (32, 34), was investigated. These enzymes were found to be inhibited by dibenzoyl ethylene and 2-hydroxy-5-methylacrylophenone to about the same extent (Table V). Urease, another example of an enzyme inactivated by compounds reacting with sulfhydryl groups (37), was also found to be inactivated by both of these ketones. The activity of a solution of crystalline urease (38), when studied by the procedure of Potter and Albaum (39), was found to be diminished 50% by  $8.0 \times 10^{-5}$  M dibenzoyl ethylene or  $8.0 \times 10^{-4}$  M 2-hydroxy-5-methylacrylophenone. It seems, then, that dibenzoyl ethylene has no special affinity for succinic dehydrogenase, but can react with the sulfhydryl groups of other enzymes as well.

#### ACKNOWLEDGMENT

The author is indebted to Dr. M. C. Fernald for supplying most of the compounds reported in Table I, the synthesis of which will be reported elsewhere, and to Dr. R. E. Lutz of the University of Virginia for his generous gift of other compounds. A large part of the bacteriological testing was performed by Miss Dorothy J. Randolph, and the tests with acid-fast bacteria by Miss Doris Hutchinson. The author gratefully acknowledges their assistance.

#### SUMMARY

1. Thirty-five unsaturated ketones were examined for the presence of antibacterial and antifungal properties. All but five showed definite antibacterial or antifungal activity.

2. Of the active compounds, 2-hydroxyacrylophenone and its derivatives, and dibenzoylethylene and its derivatives, showed the highest activity.

3. The unsaturated ketones react with mercaptans to form addition compounds, and may be partly or completely inactivated by this process.

4. The unsaturated ketones inactivate enzymes with essential sulfhydryl groups, such as succinic, alcohol and triose phosphate dehydrogenases, and urease.

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# Cyanide Inhibition of Retinal Respiration in Bicarbonate Buffer<sup>1</sup>

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Received October 27, 1947

## INTRODUCTION

An adequate explanation has not been presented to account for the difference in the cyanide inhibition of retinal metabolism in phosphate and bicarbonate buffers observed by Laser (1). He reported that the oxygen consumption of isolated rat retinas was almost completely inhibited by  $10^{-3}$  *M* cyanide in a phosphate medium, but not at all in bicarbonate-buffered Ringer's solution. Recent studies of the volatility of hydrogen cyanide gas and its loss during manometric experiments (2, 3) suggest that there may possibly have been a technical error involved in the original experiments rather than a difference in the physiological behavior of the tissue in the two media. A reinvestigation of this problem using the Summerson differential manometer technique (4) is reported below.

## MATERIALS AND METHODS

Retinas were obtained from 150–300 g. white rats after the animals had been killed by a blow on the head. The retinas were removed immediately while the eyes were in either phosphate or bicarbonate buffered saline solution (5) containing 0.2% glucose. They were then transferred quickly to micro Warburg manometer flasks or the Summerson differential manometer flasks. Carbon dioxide-absorbing solutions for the center well of the Warburg flask consisted of calcium cyanide-calcium hydroxide mixtures with hydrogen cyanide tensions equivalent to those of the experimental solutions (2). To maintain a proper concentration of cyanide in the Summerson manometer flasks, the incoming 95% oxygen-5% carbon dioxide gas mixture was passed through 2 gas-washing bottles containing neutralized potassium cyanide solution of the same concentration as the fluid in the flasks. These were kept in the water bath at 37.5°C. Results are expressed in terms of oxygen consumption per retina.

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<sup>1</sup> Aided by a grant from the John and Mary R. Markle Foundation.

## RESULTS AND DISCUSSION

Fig. 1 shows the effects of various concentrations of cyanide on the respiration of the isolated rat retina in a phosphate-buffered medium. One-thousandth molar hydrocyanic acid inhibits oxygen consumption almost completely. Fig. 2 gives the results of similar experiments with

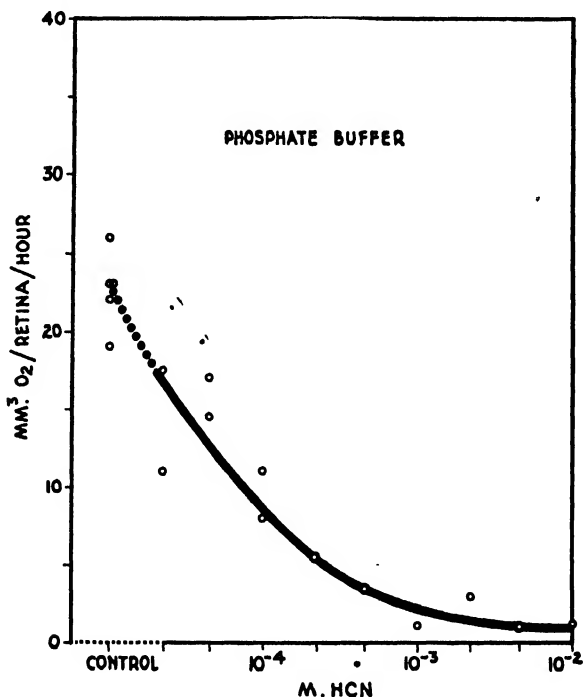


FIG. 1. Effect of various concentrations of cyanide on the respiration of rat retinas in phosphate-buffered saline solution. Calcium cyanide-calcium hydroxide center well mixtures were used to absorb carbon dioxide and maintain proper cyanide tension. Measurements were for a one hour period.

bicarbonate buffer and 95% oxygen-5% carbon dioxide gas mixture in Summerson manometers. The inhibition is approximately the same as that which occurs in the phosphate medium.

The importance of maintaining a constant concentration of hydrogen cyanide in experiments with this agent depends upon the stability of the cyanide-heavy metal complex. If the combination is only slowly dissociable the original concentration determines the effect, and escape

of cyanide during the experiment is of little importance. If the complex dissociates rapidly, a constant tension of cyanide gas is necessary in a determination of the extent of inhibition. Apparently there is considerable variation in the response of different tissues. Some cells, such as yeast, recover only slowly after the cyanide has been washed off. Other tissues, rabbit cornea for example (6), return to the original rate of respiration almost immediately after removal of the free cyanide.

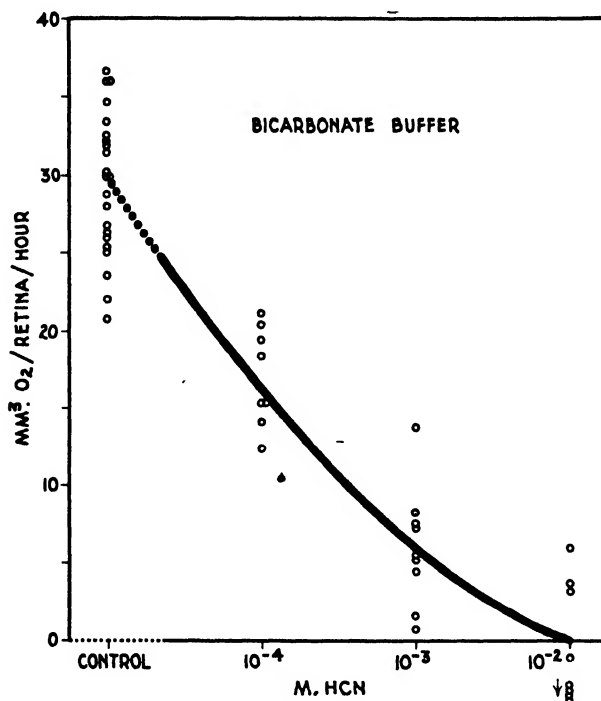


FIG. 2. Oxygen consumption of rat retinas in cyanide in bicarbonate-buffered medium. Manometer flasks were gassed with 95% oxygen-85% carbon dioxide mixture which had been bubbled through two gas washing bottles containing cyanide. Forty-five minute measurements.

The experiment illustrated by Fig. 3 indicates that the retina recovers rapidly from a short exposure to 10<sup>-3</sup> M cyanide when it is in phosphate buffer. Isolated rat retinas were placed in small Warburg manometer flasks in 10<sup>-2</sup>, 10<sup>-3</sup>, and 10<sup>-4</sup> M cyanide solution. The 10% potassium hydroxide solution in the center well rapidly absorbed the

cyanide gas given off by the experimental fluid. The decrease in concentration is reflected in the quick return to the normal level of respiration of those retinas in the two weaker solutions. The tissues in the  $10^{-2} M$  solution had still not recovered six hours later, possibly because the oxidative processes were interrupted for too long a time during the period of cyanide exposure.

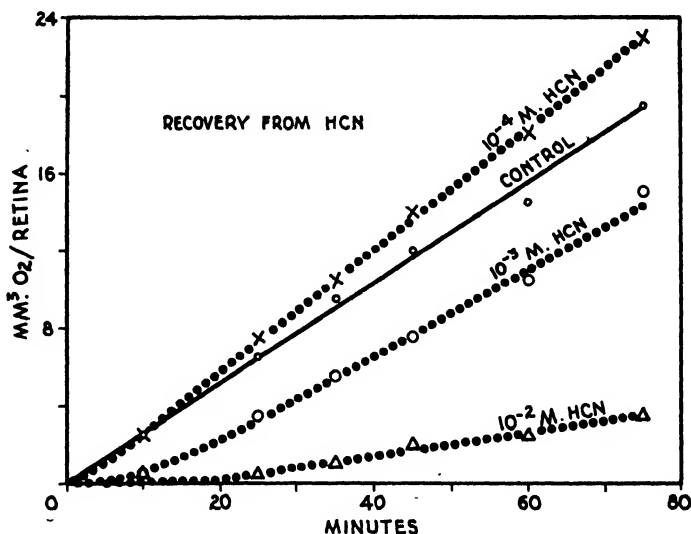


FIG. 3. Recovery of rat retinas from cyanide exposure. Cyanide placed in the experimental fluid only; the potassium hydroxide in the center wells rapidly absorbed cyanide from the experimental solution and allowed retinas in the two lower concentrations to recover.

With bicarbonate buffer in the Summerson manometer flasks, aeration with oxygen-carbon dioxide mixture containing no cyanide blows off enough hydrogen cyanide from the fluid to reduce the concentration to less than 20% of the original level (3). The retinas recover under these conditions also, and although the concentration of cyanide before gassing may have been  $10^{-3} M$  the observed respiration is almost at the control level during the subsequent measurement period. It is possible, therefore, that the lack of inhibition in bicarbonate buffer observed by Laser was due to a loss of cyanide during the aeration process. Since details of procedure are not given in the original

publication it is difficult to evaluate the adequacy of the technique. If the difference previously reported was the result of technical difficulties in controlling the concentration of cyanide, there is no justification for assuming that different respiratory mechanisms are involved when the tissues are in the different types of buffer mixtures.

The Summerson manometric technique is convenient because it makes possible simultaneous determination of respiration and glycolysis. Inhibition of the Pasteur mechanism by cyanide results in an increased rate of glycolysis. A high concentration of hydrocyanic acid will inhibit the glycolytic process, however, and lactic acid production decreases (7). The relationship of cyanide concentration to these processes is shown in Fig. 4. It may be seen that  $10^{-3}$  *M* cyanide al-

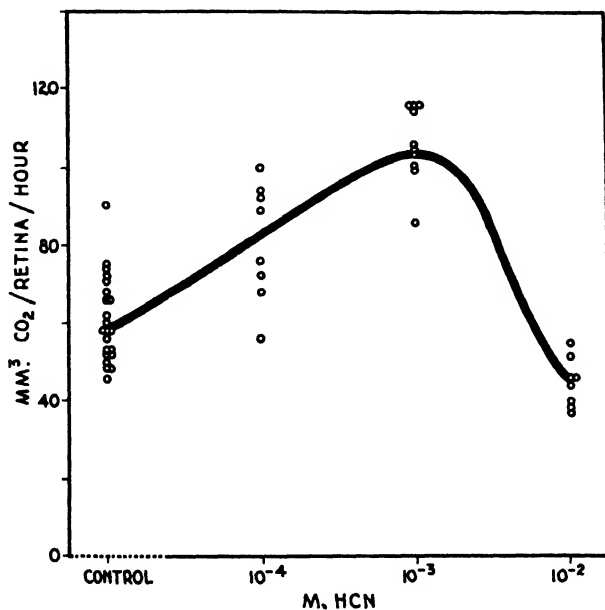


FIG. 4. Effect of cyanide on glycolysis in the rat retina. Carbon dioxide liberation measurements shown were obtained during the experiments on respiration represented in Fig. 2.

most doubles the rate of carbon dioxide release from the bicarbonate medium, but that this increase in glycolysis is completely inhibited by a concentration of  $10^{-2}$  *M* cyanide.

## SUMMARY

Respiration of the isolated rat retina is almost completely inhibited by  $10^{-3}$  *M* hydrocyanic acid in either phosphate- or bicarbonate-buffered medium. Recovery from a short exposure to this concentration of cyanide is rapid, and a previous report which indicated that the retina was insensitive to cyanide in a bicarbonate medium may have been in error because of loss of cyanide during the gassing of the manometers.

The rate of glycolysis is doubled by  $10^{-3}$  *M* cyanide, but returns to the control level or below at  $10^{-2}$  *M* cyanide.

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# Enzymes of Fresh Hen Eggs

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Received September 17, 1947

## INTRODUCTION

The work reported in this paper was stimulated by the need for information relating to the deterioration of dehydrated eggs. Although enzymes have not been shown to cause any of the observed chemical changes that occur in the deterioration of eggs, it has been suggested that certain deteriorative changes might be enzyme-catalyzed (1). On the other hand, some workers have felt that the small amount of enzyme activity exhibited by eggs indicates that enzymes are not of primary importance in dehydrated egg deterioration. Since there is disagreement regarding the presence of certain enzymes in eggs and since some of the earlier workers on this subject employed techniques now recognized as unreliable, it appears that a better knowledge of the enzyme content of eggs is required to evaluate the potential role of constituent enzymes in the deterioration of liquid, frozen, and dried eggs.

Most of the work on egg enzymes has been related to embryology, and has been adequately reviewed by Needham (2, 3). Enzymes that have been reported to be present in unincubated eggs are catalase (4, 5), cholinesterase (6), erepsin (7, 8, 9), lipase (5, 7), ovomucoidase (10), phosphatase (11), procaine esterase (12), protease (7, 13), amylase, histozyme, lecithinase, oxidase, and salicylase (7). Although glycolysis (2) has been reported to occur in fresh eggs, no respiration of either whole egg or the egg fractions has been detected (14).

Although the work reported here was undertaken to obtain quantitative data on egg enzyme activities that would be of value in interpreting observed changes in egg material, the results on this preembryonic material should also be of general biochemical interest. Selection of

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enzymes for study was influenced by both the limited egg enzyme literature (2, 3) and the changes that are known to occur in egg substance (1, 15, 16). The enzyme content has been determined by measurement of activity under favorable assay conditions. Enzyme activities under the assay conditions would generally, and perhaps always, be much greater than under the less favorable autolytic conditions. Each of the following enzymes or class of enzymes is briefly reviewed in the section of the paper dealing with the particular enzyme: Esterases (tributyrylase, lipase, phosphatase), peptidases (erepsin), catalase, oxidase, cytochrome oxidase, peroxidase, amylase, and proteinase.

#### GENERAL CONSIDERATION OF MATERIALS, METHODS, AND UNITS USED TO EXPRESS ACTIVITIES

The eggs used in this study were, in general, eggs of known history. Some of the preliminary experiments were made on eggs purchased as "fresh" on the open market, but, in all cases, the final experiments were conducted on eggs from one to a few days of age that were refrigerated after the first 24 hours until used. The data for some enzymes were obtained on eggs known to be infertile. This fact, as well as other pertinent information relative to a particular enzyme, is given in the section dealing with that enzyme.

When egg yolk and egg white were used separately, care was taken to avoid contaminating one with the other. In preparing yolk, the white on the surface of the yolk was removed by washing in water before the yolk membrane was broken. Livetin, which was used in several experiments, was prepared from yolk that had been dried by lyophilization. The residue from ether extraction of the dried yolk was suspended in distilled water and the insoluble lipovitellin removed by centrifugation. The supernatant solution was dialyzed, concentrated *in vacuo*, and lyophilized. The livetin fraction prepared in this way represents 6-7% of the total yolk solids.

Whereas no attempt was made to maintain aseptic conditions, care was taken to avoid gross contamination. Only unsoiled eggs were used, and these were thoroughly cleaned by scrubbing with a good detergent. In addition, the eggs used in the long-time experiments were dipped in alcohol and flamed immediately before breaking.

Compared with the enzyme activity in materials recognized as good sources of enzymes, the egg-enzyme activities were small. The low order of activities made it essential to estimate carefully the sensitivity and precision of assay methods. This care was especially required for enzymes that were absent within experimental error. No effort was spared in guarding against false activities due to microorganisms, or unstable substrates. The effectiveness of preservatives was determined under conditions of all assays. Toluene, so frequently used as a preservative in enzyme work, was found to be woefully inadequate with egg yolk. The fact that yolk is an excellent medium for bacterial growth (17) probably accounts, at least in part, for the limited value of toluene, since it is recognized that bacterial growth is difficult to control under favorable growing conditions (18). The limitations of toluene have also been

pointed out by Bamann and Myrbäck (18) and by Schultze (9). A combination of toluene and chloroform, also described by Bamann and Myrbäck, was found to be an effective preservative under some conditions. Merthiolate in concentrations of 0.05–0.10% was also an effective preservative for this work and has been used in most of the experiments. The details of the work on preservatives are given on page 446.

To place reliable limits on enzyme content, two types of control experiments were run for most assays. These were the complete reaction mixture less the enzyme source, and the complete reaction mixture less the substrate. The substrates under the assay conditions were quite unstable in some cases. This was particularly true in the oxidase work at higher pH values. The reported activities have been corrected when the controls exhibited any change.

All enzyme activities are expressed on a comparable basis (19), with the exception of catalase activity. The unit of activity is the amount of enzyme required to alter one milliequivalent of substrate per minute, and the values are expressed per kilogram of dry weight. Catalase activities are expressed, as is customarily done, as the first order *K* value, or *Katalase Fähigkeit* (*Kat. F.*).

#### AUTOLIPOLYSIS

The presence of large amounts of lipoidal substances in hen eggs, particularly triglycerides and phospholipides, lends special significance to the investigation of lipolytic action on these natural substrates by inherent enzymes. The results of Wohlgemuth (20) and Koga (7), purportedly demonstrating the degradation of both triglyceride and phospholipide, are generally cited as evidence for the presence of lipolytic enzymes in eggs. Their evidence led Brooks (1) to postulate that the slow increases in free fatty acids and phosphoric acid, which arise during the storage of shell eggs or dehydrated eggs, may be attributed to the action of inherent enzymes on these natural substrates. Since the magnitude of these activities is exceedingly small, their measurement in long-time experiments is complicated by such factors as microbial development, CO<sub>2</sub> absorption, and perhaps even appreciable chemical hydrolysis. With these factors taken into consideration, an examination of the data presented by Wohlgemuth and by Koga indicates the dubious validity of their conclusions.

Wohlgemuth incubated egg-yolk melange, containing about 1% added toluene, at 38°C. during a period of 1–2 months and construed the qualitative identification of glycerine, free phosphoric acid, and choline as evidence for autolipolysis. Tests for microbial contamination apparently were limited to occasional olfactory examination for putrefaction. In addition to the lack of microbial controls, Wohlgemuth's results were so variable as to arouse suspicion. Thus, in 8 experiments with egg yolk, 3 resulted negatively; i.e., no indication of lipolytic activity was detected. He attributed this inconsistency to the possible inhibitory influence of the preservative used, stating that

excess toluene, or the use of chloroform in addition, often resulted in only weak autolysis or none. While it appears that Wohlgemuth's results could be more logically attributed to the effects of microbial growth, he did, however, report a single experiment in which whole-egg melange, prepared aseptically and incubated for two weeks, gave positive tests for choline and free phosphoric acid. Bouillon and agar cultures made at the start and conclusion of this experiment indicated that organisms were not present.

Koga's evidence for lecithinase activity in hen eggs is based on the development of acidity in 1% yolk emulsions containing "some" toluene and incubated for 24 hours. The increase in titratable acidity (phenolphthalein end point) in 5 replicate experiments amounted to 25–55 ml. *N*/10 NaOH solution/100 ml. yolk. The variable nature of Wohlgemuth's and Koga's results, plus the finding that toluene is not a reliable preservative for egg-yolk medium, makes the conclusions of those investigators appear to be uncertain. The following results show that autolipolysis does not occur within experimental error of measurement in the absence of microbial activity.

### *Methods*

Since the reports of Koga and Wohlgemuth on the autolipolysis of egg yolk were based on the use of a preservative of questionable efficiency, their experiments were essentially duplicated using several preservatives in order to evaluate the efficacy of toluene as used by them, and to determine whether acid production or lipolysis could occur independently of microbial contamination. The experimental set-up is evident from Table I. Two runs were made without any preservative for comparison with the results obtained in the presence of the preservatives. One of these runs was agitated by passing nitrogen (containing 0.6% oxygen) through the yolk. This was done to reduce the amount of oxygen available for growth of aerobic organisms. In this experiment, the shells of the eggs were treated by igniting alcohol on their surfaces before breaking, but no other attempt at asepsis was made, although special care was taken to avoid gross contamination. The yolks from several dozen fresh eggs were washed with distilled water, mixed with three parts of freshly boiled distilled water, and filtered through a milk filter. Three hundred ml. of diluted melange was placed in each of six 500-ml. stoppered flasks. Gas that was bubbled through some of the reaction mixtures during the course of the experiment was first passed through boric acid solution and soda lime to remove traces of  $\text{NH}_3$  and  $\text{CO}_2$ . Samples were removed for analysis at zero time as well as at the time intervals indicated in Table I. The efficiencies of the preservatives were followed by making plate counts according to accepted methods (21). The titratable acidity was determined by titrating duplicate 5 ml. aliquots from the existent pH to pH 9.0 as measured on the Beckmann pH meter. Ten ml. aliquots, removed for the free fatty acid determination, were frozen immediately and dried in the frozen state. Free fatty acids were determined when convenient, according to the method of Johnson and Kline (22). Volatile acids were determined on 10 ml. aliquots that were removed, frozen immediately, and stored at  $-30^\circ\text{F}$ . until assayed. The assay was carried out by rapidly thawing the sample, adding 10 ml. of a phosphoric acid solution designed to adjust the pH to 2.4, collecting 100 ml. of steam distillate at constant still volume and titrating the distillate to a prepared standard phenolphthalein pink with *N*/50 NaOH solution. Increases over the initial values were calculated to meq./kg. of dry yolk.

TABLE I

*The Effect of Preservatives on the Development of Microorganisms  
and Acidity in Egg Yolk Mixtures*

Preservative	Gas bubbled through	Reac- tion time	Plate count <sup>1</sup>	Increases/kg. yolk solids in meq. <sup>1</sup>		
				Titratable acidity <sup>1</sup>	Free fatty acids <sup>1</sup>	Volatile fatty acids <sup>1</sup>
None	None	<i>Hrs.</i> 7	<i>colonies/ml.</i> $4.6 \times 10^5$	0	0	
		24	$2.9 \times 10^8$	110	60	
		48	$>4 \times 10^8$	280	210	
2% toluene	None	7	0	0	0	
		24	$0.6 \times 10^8$	28	9	7
		48	$0.3 \times 10^8$	130	110	20
2% touene	Air	7	0	0	0	
		24	$0.3 \times 10^8$	0	0	
		48	$>5 \times 10^8$	0	0	
0.1% merthiolate	Air	7	0	0	0	0
		24	0	0	0	0
		48	0	0	0	0
0.5% sodium pro- pionate	Air	7	0	0	0	
		24	$>5 \times 10^8$	18	0	
		48	$2.3 \times 10^8$	-12	0	
None	N <sub>2</sub>	7	$1 \times 10^4$	0	0	0
		24	$1.6 \times 10^8$	280	50	14
		48	$>4 \times 10^8$	410	160	60

<sup>1</sup> Zero time values:

Plate count.....	0
Titratable acidity.....	120
Free fatty acids.....	15
Volatile fatty acids.....	0

### *Results and Discussion*

Two significant results of this experiment are immediately apparent. First, the inclusion of 2% toluene in the yolk suspension, either in the quiescent state or agitated by bubbling air, does not prevent microbial

growth (Table I). Second, increase in titratable acidity occurs only in those cases where microbial contamination is demonstrated. Moreover, the development of acidity was not linear, being negligible in 7 hours and increasing rapidly between 7 and 24 hours. In addition, volatile fatty acids, normally not present in fresh egg yolk either free or esterified, appear as part of the developed acidity. Merthiolate appeared to be the only completely effective preservative used in this experiment, although both toluene and sodium propionate did retard the development of contamination.<sup>2</sup> No attempt was made to balance the net development of acidity, since lactic and other essentially nonvolatile acids were not determined nor was a correction made for the liberation of ammonia or free amino groups, which would be partially titrated at pH 9.0. In those cases where air was supplied, microbial growth was not accompanied by an increase in the titratable acidity. The facts that the acidity increased only when low oxygen tension was present and that both volatile fatty acids and water-soluble acids (*e.g.*, lactic acid) developed to a considerable extent, particularly in the case where nitrogen was passed through the yolk suspension, suggest the occurrence of both the "mixed acid" and lactic acid type fermentations characteristically produced by many facultative anaerobes. The growth of lipolytic organisms was apparent, also, from the free fatty acid increases.

### *Summary*

No autolipolysis has been detected in egg yolk substance in the presence of adequate preservatives. Evidence has not been found, therefore, to support the contention that inherent egg enzymes are responsible for reported increases in acidity of yolk materials. Significant changes in acidity may occur, however, in the presence of contaminating organisms, particularly under conditions of low oxygen tension.

### ESTERASES

Pennington and Robertson (5) reported increases in titratable acidity when whole-egg melange was incubated with ethyl butyrate. However, they used toluene as a preservative in experiments of several days'

<sup>2</sup> In another experiment a combination of 2% toluene and 1.2% chloroform was found to be an adequate preservative, even though neither of these substances alone was satisfactory. This is in agreement with the literature (18).

duration and it is, therefore, difficult to place a quantitative interpretation on their results.

Koga (7) compared the activities of yolk and white emulsions incubated with monobutyryn for 24 hours, by determining the increase in titratable acidity. On an equal volume basis the yolk was reported to contain about 4 times the activity of the white. Although "some" toluene was used as preservative, the results were variable and, again, cannot be construed with safety as an indication of the relative activities of the yolk and white on monobutyryn. Koga also compared tributyrinase activities of white and yolk by the stalagmometric method of Rona and Michaelis (23), which depends upon the decrease in surface tension attending the hydrolysis of the substrate. He reported that there was considerably more tributyrinase in the white than in the yolk, in spite of the fact that again he obtained results that varied widely and, indeed, obtained negative results occasionally with egg white. In contrast with Koga, Ammon and Schütte (6) found several-fold more tributyrinase in the yolk than in the white. Ammon and Schütte also reported that the rate of hydrolysis of methylbutyrate and acetylcholine by egg yolk was greater than by egg white. Their work appears to be precise; liberation of acid as indicated by  $\text{CO}_2$  evolution (Warburg technique) was usually linear in experiments of a few hours' duration, and adequate controls to account for any chemical hydrolysis of substrates were carried out.

### *Method*

The initial experiments to determine the magnitude and distribution of esterase activity in eggs were carried out with tributyrin according to the method of Mattick and Kay (24). Five g. of whole egg powder, or dried yolk or white, were emulsified with 0.5 ml. of tributyrin and 100 ml. of *N*/10 veronal buffer at pH 8.5 on a shaking machine, and the mixture was placed in a 37°C. water bath. At suitable intervals, usually 1 hour, 20 ml. aliquots were removed and acidified to pH 2.4, with 0.5 ml. of a 42%  $\text{H}_3\text{PO}_4$  solution. The free butyric acid was collected by steam distillation in 100 ml. of distillate, while the volume of the sample was kept constant. The distillate was titrated with *N*/50 NaOH solution to a prepared standard phenolphthalein pink. The rate of liberation of butyric acid, which was constant for at least 4 hours, was obtained from the slope of the line resulting from the plot of the titer values as a function of time. For comparing the rates on various substrates, the continuous neutralization procedure as described under the section on lipase was used. With materials of sufficient activity, the two methods agree. The first method is the more sensitive one and was resorted to primarily with samples of comparatively low tributyrinase activity.

### *Results and Discussion*

The tributyrinase activities for whole dried eggs, either of commercial origin or prepared from fresh eggs in the laboratory by lyophilization, were found to be sufficiently uniform (Table II) to preclude microbial origin of this activity. Preservatives (toluene, chloroform, merthio-

TABLE II  
*Tributyrylase Activity of Eggs, Whole Milk, and Pancreatin*

Material	Units/kg. dry weight
Distribution in whole egg	
Commercial spray-dried eggs (7 samples)	0.31-0.35
Lyophilized fresh eggs <sup>1</sup> (8 batches)	0.28-0.34
Lyophilized egg yolk (8 batches)	0.38-0.46
Lyophilized egg white (8 batches)	0.09-0.14
Distribution in yolk	
Ether-soluble fat	0
Lipovitellin	<0.10
Yolk membrane	0.30
Livetin	4.6-5.8.
Milk and pancreatic tributyrinase	
Lyophilized skim milk	3.7
Whole milk, Mattick and Kay <sup>2</sup> (24)	0.8-2.3 (calculated)
Whole milk, Peterson <i>et al.</i> (25).	1.5-6.9 (calculated)
Pancreatin I (Commercial U.S.P.)	$2 \times 10^3$
Pancreatin II (Commercial U.S.P.)	$17 \times 10^3$

<sup>1</sup> Measurement of whole egg, egg yolk, and egg white tributyrinase activities on representative samples from a large batch of 15 dozen fresh eggs revealed activities, on a dry-weight basis, of 0.30, 0.40, and 0.10, respectively. The percentage of the total activity of whole eggs residing in the yolk was calculated to be about 87%.

late, and thymol) added to the whole egg digestion mixture were without effect on the activity. The enzymic nature of the activity was evidenced by its heat lability. It was destroyed by heating either the 5% egg digestion mixture at 62°C. for 0.5 hour, or whole-egg melange at 70°C. for the same time. The energy of activation of destruction was about 42 kg.-cal. (Fig. 1). Contrary to the findings of Koga, but similar to those of Ammon and Schütte, about 87% of the tributyrinase activity of the whole egg was found in the yolk. Recalculation of the results of Ammon and Schütte from their given "G" values for the total tributyrinase content per yolk or white, gave values per kg. dry weight of 0.48 unit for the yolk and 0.10 unit for the white. Our values (Table II) ranged from 0.38 to 0.46 unit for yolk and 0.09 to 0.14 unit for white.

It was found that the livetin fraction, which represented 6-7% of the yolk solids, retains at least 90% of the total yolk tributyrinase activity, and therefore represents a 14- or 15-fold concentration of tributyrinase.

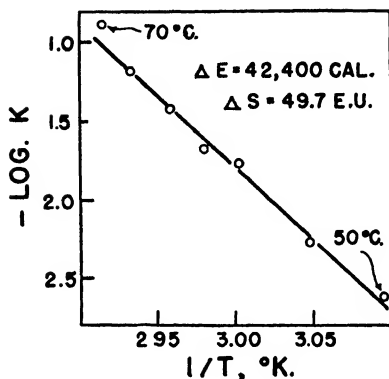


FIG. 1. Inactivation of tributyrinase in whole-egg melange. The  $K$  for inactivation was calculated from the residual activity after 30 minutes of heating on the assumption that inactivation was a first order reaction.

As a very rough approximation, compared on a dry basis, the tributyrinase activity of whole egg was about 1/10 that of milk and about 1/10,000 that of commercial pancreatin. The pII optimum of the yolk tributyrinase (*ca.* pH 8.5, Fig. 2) is similar to that reported for milk tributyrinase (24, 25).

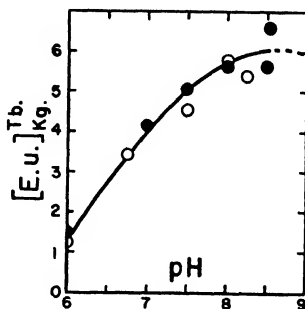


FIG. 2. Activity-pH curve for tributyrinase activity of egg livetin.

The relative activities of livetin on various substrates are given in Table III. Detectable activity ceases abruptly as the chain length of the fatty acids of the esters is increased to 6 or more carbons. Thus the esterase activity of egg, like that of liver, is restricted to esters of the lower fatty acids.



TABLE III  
*Activity of Egg Livetin on Various Substrates*

Substrate	Concentration of substrate		pH	Units/kg. dry weight
	Normality <sup>1</sup>	Per cent		
Methylbutyrate	0.17	1.7	8.0	3.5 <sup>2</sup>
Benzylbutyrate	0.11	2.0	8.0	3.8
Acetylcholine	0.11	1.8	7.5	8.8 <sup>2</sup>
Acetylcholine	0.02	0.3	7.5	8.7
Benzoylcholine	0.03	0.7	7.5	0.3
Acetyl- $\beta$ -methyleholine	0.02	0.4	7.5	2.0
Triacetin	0.16	1.2	7.5	1.7
Tripionin	0.20	1.7	8.0	7.4
Tributylin	0.20	2.0	8.0	5.6
Tricaproin, egg oil, } olive oil, etc. }		1.5	8.0	<0.02

<sup>1</sup> Normality refers to equivalents of ester bonds/l.

<sup>2</sup> Calculation of Ammon and Schütte's (6) results to a kg. dry weight of yolk basis gave values of 0.25 unit and 0.53 unit, respectively, for the substrates methyl butyrate and acetylcholine. Our results for livetin, converted back to yolk solid basis by dividing by 14, gave values of 0.25 unit for methylbutyrate and 0.62 unit for acetylcholine. /

Procaine esterase was reported to be present in egg white by Kirsch *et al.* (12). Attempts to measure this activity by our technique, which requires greater substrate concentrations than they used, were inconclusive because of high rate of chemical hydrolysis at these concentrations.

Limited studies of inhibition by eserine showed that egg tributyrinase, unlike liver esterase and mammalian plasma esterase (26), was completely inhibited by  $10^{-5}$  M eserine. Also, under our assay conditions, pancreatin tributyrinase was about 30% inhibited and milk esterase was 50–80% inhibited by the above eserine concentration. Thus, egg tributyrinase appears to be distinct from the common esterases as far as eserine inhibition is concerned, although it is similar to liver as far as specificity is concerned.

The cholinesterase of livetin appears to differ from mammalian cholinesterase as described by Mendel *et al.* (26). The slow rate of hydrolysis of benzoylcholine indicates the presence of very little

"pseudo"-cholinesterase, yet the rate of hydrolysis of acetyl- $\beta$ -methylcholine is not sufficient to account for more than 50% of the rate of acetylcholine hydrolysis (*cf.* Mendel *et al.*, 26). In respect to relative rates of hydrolysis of acetylcholine and acetyl- $\beta$ -methylcholine, the esterase resembles planaria cholinesterase (27) more than mammalian cholinesterase. The inhibition of egg cholinesterase by  $10^{-5}$  *M* eserine, as first reported by Ammon and Schütte, has been confirmed.

### Summary

Per kg. dry weight, egg yolk contains about 0.4 unit and egg white about 0.1 unit of tributyrinase activity. The tributyrinase activity of the yolk is almost quantitatively associated with the livetin fraction and is completely inhibited by  $10^{-5}$  *M* eserine. The livetin fraction contains, in addition to tributyrinase activity, activity toward esters of other short-chain (less than six carbons) fatty acids, none of which is known to occur in eggs.

### LIPASE

Even though no lipolysis has been found to occur in egg yolk substance (Table I), the fact that yolk has tributyrinase activity as well as activity toward esters of other short-chain fatty acids, stimulated additional work on egg lipase. Since the fatty acids of egg are almost exclusively restricted to those containing 16 carbons or more (28,29), tests were made on natural and modified egg fats and on other fats that contain long-chain fatty acids. Crude egg fractions, as well as livetin, which contains most of the yolk tributyrinase, were tested for lipase activity.

### Method

The enzyme activities were determined by the continuous titration procedure as described by Lineweaver and Ballou (19). The reaction mixture of 20 ml. contained the enzyme preparation (1.0 g. dry whole egg or yolk, or 0.5 g. livetin, or 2.5 mg. commercial U.S.P. pancreatin), 0.35 ml. of olive oil, egg oil, *etc.*, added in 3.5 ml. of a bile-glycerol emulsion prepared according to Balls *et al.* (30), and NaCl (0.1 *M*). In addition, 0.03 *M*  $\text{CaCl}_2$  was used as an activator in all cases where its presence did not destroy the emulsion.  $\text{CO}_2$ -free air was continuously passed through, or in some cases over, the reaction vessel, which was supported in a 37°C. water bath. A pH of 8.0 was maintained by adding *N*/50 NaOH solution to neutralize any acidity which developed.

*Results and Discussion*

The maximum values of 0.02 unit/kg. for livetin and 0.002 unit/kg. dry weight for whole egg or yolk (Table IV) are approximate figures

TABLE IV  
*Lipase Activity on Glycerides of Long-Chain Fatty Acids*

Enzyme	Substrate	Units/kg. dry weight
Whole yolk solids	Yolk	<0.002
Whole yolk solids	Olive oil	<0.002
Whole egg solids	Olive oil	<0.002
Whole yolk solids	Tricaproin	<0.002
Livetin	Whole yolk fat	<0.02
Livetin	Olive oil	<0.02
Livetin	Tricaproin	<0.02
Livetin	Yolk fat with phospho- lipides removed	<0.02
Pancreatin (U.S.P.)	Yolk solids	$3.4 \times 10^2$
Pancreatin (U.S.P.)	Olive oil	$3.0 \times 10^2$
Pancreatin (U.S.P.)	Yolk fat with phospho- lipides removed	$1.5 \times 10^2$

indicating the experimental error as estimated from controls on the substrate or enzyme alone. These results, considered along with those reported in Tables I and II, show that the lipolytic activity of eggs, like that of liver, is largely, if not entirely, restricted to esters of short-chain fatty acids. Pancreatin, on the other hand, as reported by Balls and Matlack (31), has activities of a similar order of magnitude on triglycerides of both short chain (*cf.* tributyrin) and long chain fatty acids.

## PHOSPHATASES

Free phosphate, which is slowly liberated during storage of eggs (1), generally has been assumed to arise from decomposition of the phospholipids, but may also arise from the phosphoprotein that occurs in the vitellin fraction of the yolk.

Recently, Harris (32) reported the presence of a specific phosphoprotein phosphatase in the yolk material of frog eggs that acts on the phosphoproteins of the yolk material, on hen egg vitellin, and on casein. Organic acid-soluble phosphorus and lipid phosphorus of yolk remained essentially unchanged. However, in a single experiment, Harris did not find similar activity in hen eggs. Mecham and Olcott, in this laboratory, have isolated from the vitellin fraction of egg yolk a phosphoprotein containing 10% phosphorus. This protein is readily hydrolyzed by vegetable phos-

phatase (33). Thompson (11) in 1943, using phenyl phosphate as substrate, reported the presence of an alkaline phosphatase in egg.

### *Method*

Thompson's procedure (11) was followed with regard to buffer, temperature, pH, method of preparing the trichloroacetic acid filtrate, and method of phenol determination, but one-hour reaction times were used instead of 3 hours. With glycerophosphate as substrate, the liberated phosphate was estimated by Allen's method (34). The phosphatase content of skim-milk powder, dried from unpasteurized skim milk in this laboratory, was determined by the usual method of determining milk phosphatase. In Thompson's procedure, the reaction mixtures are adjusted to pH 9.0 and incubated at 37°C., while, for the milk phosphate method, the mixture is adjusted to 9.6 and incubated at 47°C.

### *Results and Discussion*

The complex nature of the yolk material unfortunately presented the difficulty of measuring small increments in the presence of high blank values. However, the results, although variable, were sufficiently consistent to obtain an approximate measure of the magnitude of the yolk phosphatase activity (Table V). Thompson's results, recalculated

TABLE V  
*Phosphatase Activity of Egg Yolk*

Enzyme	Substrate	Units/kg. dry weight
Egg yolk	M/200 Phenylphosphate	0.018-0.046
Egg yolk	M/20 Phenylphosphate	0.060
Egg yolk	M/20 Phenylphosphate	0.004-0.020 <sup>1</sup>
Egg yolk	M/20 Glycerophosphate	0.046
Skim milk	M/400 Phenylphosphate	1.1-2.2

<sup>1</sup> Thompson's results (11).

in terms of our units, are about one-fourth as great as those we obtained. Like Thompson, we detected no phosphatase in egg white. It appears that yolk-phosphatase activity on a dry-weight basis is about 1/20 of the phosphatase activity of skim-milk powder, and is about 1/10 the order of activity of yolk tributyrinase. Unlike tributyrinase, less than 1/5 of the total quantity of yolk phosphatase was recovered in the livetin fraction.

No activity was found toward substances normally present in egg yolk, since the free phosphate content of yolk substance without added substrate did not change.

### *Summary*

Egg yolk contains, per kg. dry weight, only 0.05 unit of alkaline phosphatase activity toward simple phosphate esters. No activity was detected toward naturally occurring substances. No phosphatase activity was found in egg white.

### PEPTIDASES

Peptidases (erepsin) have been reported in egg yolk by Koga (7), and in egg white by Van Manen and Rimington (8) and Schultze (9). The results of these investigators have not been challenged so far as the writers are aware. A critical study of their methods and results shows that the data of Koga and of Van Manen and Rimington, while qualitatively probably reliable, leave much to be desired from a quantitative viewpoint. Schultze's work is not subject to this criticism. Results on effectiveness of preservatives, reported in this paper, throw doubt on Koga's results, since toluene does not adequately prevent microbial contamination. Also, Koga used a color "end-point," which is not precise in the presence of yolk. Van Manen and Rimington likewise used toluene in their work, but, since their enzyme reaction mixture was much less favorable for bacterial growth than is the case with egg-yolk studies, they probably had little microbial interference. On the other hand, they used a method that does not quantitatively account for the free amino groups. Furthermore, their results were not linear with respect to time, and, in addition, even their 1-3 hour values showed about a 15-fold variation from experiment to experiment.

### *Method*

The yolks from nonfertile eggs were separated from most of the white and washed in running water to aid in removal of the last traces of white. After drying with a soft cloth, the yolks were diluted with an equal volume of water, stirred thoroughly to give a homogeneous preparation, and strained through a milk filter. The pH was adjusted to the desired value by addition of 0.5 *N* acid or alkali solution.

The whites of nonfertile eggs were squeezed through milk filters 3 times to facilitate the breaking up of the thick portion. An equal volume of CO<sub>2</sub>-free water was added and the pH value adjusted as desired by additions of 0.5 *N* HCl solution.

The complete enzyme reaction mixture consisted of 20 ml. of diluted egg yolk or diluted egg white as desired, 16 ml. of 2% Witte peptone solution, 4 ml. of 0.1 *M* phosphate buffer, and merthiolate to 0.05% final concentration. (See fifth paragraph under section on "General Considerations, etc." for controls used.) To maintain a more homogeneous solution when yolk was used, the reaction mixture was agitated mildly with sterile CO<sub>2</sub>-free air. Aeration was not required in experiments with egg white and, in fact, had aeration been used, it would have caused pH changes due to removal of dissolved CO<sub>2</sub>, which is present in considerable amounts in the white. The rate of change in free amino group content was followed by titrating duplicate 5-ml. aliquots initially and after 3-hour and 6-hour intervals.

Titration was carried out as follows: 5 ml. aliquots of the reaction mixtures were diluted with 5 ml. of 1% NaCl solution in a 50-ml. beaker, which was then placed on a

special turntable<sup>3</sup> arranged so that the beaker could be rotated around an electrode assembly of the pH meter. After the initial pH value was noted, the solution was titrated to pH 7.00; a 3 ml. sample of neutralized U.S.P. formaldehyde was then added, and titration was continued to pH 9.00 with 0.05 *N* NaOH solution. The titer value from pH 7.00 to pH 9.00, less the titer value for 3 ml. of formaldehyde in 5 ml. of 1% NaCl solution, represents the free amino group content.

### Results

The egg-yolk peptidase activity values in Table VI represent the average of duplicate aliquots for a single run. Maximum activity was obtained at about pH 6.7 with somewhat lower activity at pH 6.1 and

TABLE VI  
*Peptidase Activity of Hen Eggs*

Number of eggs composing sample	pH of reaction mixture	Units/kg. dry wt.			
		White <sup>1</sup>		Yolk	
		3 hrs.	6 hrs.	3 hrs.	6 hrs.
1	6.7±0.2	0.16	0.13	0.15	0.11
1	6.7±0.2	0.17	0.19	0.12	0.09
1	6.7±0.2	0.16	0.20	0.11	0.09
1	6.7±0.2			0.10	0.09
3	6.7±0.2	0.11 Tk	0.13 Tk	0.11	0.09
3	6.7±0.2	0.16 Tn	0.12 Tn	0.10	0.10
3	6.7±0.2	0.13 Tk	0.10 Tk	0.11	0.11
3	6.7±0.2	0.11 Tn	0.14 Tn		
	Average	0.15	0.14	0.11	0.10
1	8.0	0.14	0.18		
1	8.1	0.12	0.10		
1	8.1	0.12	0.21		
3	6.1			0.09	0.07
3	7.0			0.05	0.05
3	8.0			0.04	0.04

<sup>1</sup> All values are for whole egg white except those marked Tk and Tn, which are for thick and thin white, respectively.

<sup>3</sup> A beaker holder made from a No. 10 one-hole rubber stopper was placed on the vertical shaft of a constant-speed stirring motor. This motor was mounted so that it could be easily lowered and raised to facilitate handling of the sample.

significantly lower values at pH 7.0 and above; however, a detailed study of the pH-activity relationship was not made. Less than a 2-fold variation in activity was encountered between samples, even though some samples represented only individual eggs. The good agreement between the activity values obtained at 3 and 6 hours (0.11 and 0.10 unit/kg. at pH 6.7) shows that the rate was quite constant for at least 6 hours.

The egg white peptidase values (Table VI) varied about 2-fold and averaged 0.15 and 0.14 unit/kg. dry weight for the 3- and 6-hour observations, respectively. In general, the activity remained constant for 6 hours, although a few of the samples showed fluctuations. It is probable that these were due largely to the error of the method, since the  $\Delta$  titer values amounted to only about 0.10 ml./3-hour interval. The data for the thin and thick white are in good agreement, whereas Van Manen and Rimington reported the thick to be much more active than the thin. Also in contrast with the results of Van Manen and Rimington, who reported optima for egg white at pH 5.5 and 7.0-8.0, no optima were encountered at the pH values used in this study. As previously mentioned, their results showed about a 15-fold variation, while only a 2-fold variation was encountered in this study.

### *Discussion*

Earlier reports of the presence of peptidase in both the yolk and white of hen eggs have been confirmed. Most of the earlier work was not satisfactory from a quantitative standpoint and, since the yolk and white were not studied by the same workers, the relative activities of these fractions were uncertain. In the work reported here both yolk and white were studied under comparable conditions and the quantitative character of the data allows a direct comparison to be made of the relative activities of these two fractions. On a fresh-volume basis the yolk is about 2.5 times as active as the white, but, on a dry-weight basis, the yolk is only about 2/3 as active as the white.

For comparison, the results of other investigators have been calculated in terms of the units used here. Even though there are reasons, as stated elsewhere, why some of these data should not be considered in a quantitative light, nevertheless, it may be of interest to compare the order of magnitude of the results obtained by different workers. These data, along with the pertinent assay conditions, are shown in Table VII. Schultze's values are about 1/3 as much as ours, and Van

TABLE VII  
*Conditions and Results of Peptidase Assays of Hen Egg Fractions by Several Investigators*

Investigators	Temp. °C.	Preservative	pH value	Peptone conc.	Egg fraction			Reaction time hrs.	Unit/kg. dry wt.	Method
					Reaction mixture conc.	In aliquot				
						Per cent	ml.			
White										
Van Manen and Rimington	37	0.25% toluene	5.5 to 8.4	0.57	8.6	0.85	1-3 20-24 48	0.70 0.10 0.08	Sorensen's formol titra- tion, using 0.1 N NaOH solution	
Schultze	40	0.02% organic mercury com- pound related to merthiolate plus some toluene	8.5	2.7	20.	2.50	20 44	0.05 0.05	Titrated in alcoholic medium using 0.05 N sodium ethylate	
This work	37	0.05% merthiolate	6.7	0.8	25.	1.25	3 6	0.15 0.14	Titrated from pH 7.00 to pH 9.00 in presence of about 9% CH <sub>3</sub> O using a potentiometer for ob- serving pH value.	
Yolk										
This work	37	0.05% merthiolate	6.7	0.8	25.	1.25	3 6	0.11 0.10	Modified Sorensen for- mol titration using 0.2 N Ba(OH) <sub>2</sub> solution	
Koga	37	toluene	not speci- fied	1.0	25	5.00	24 48	0.08 0.07		



Manen's and Rimington's are 0.5-5 times as much, depending on the reaction time considered. Koga's value on egg yolk is about the same as ours, even though his reaction mixtures were probably not adequately preserved.

The results reported here show that the peptidase activity of hen eggs, while real, is very low in comparison with the activity that would be expected in a good source of peptidase. Consideration of the small amount of activity, along with the fact that eggs contain no known substrates for this enzyme, makes it highly improbable that peptidases play a part in egg deterioration.

### *Summary*

The peptidase activity of both the yolk and white of hen eggs has been quantitatively determined. No difference in activity between thin and thick white was detectable. Per kg. dry weight, the yolk and white contain 0.11 and 0.15 unit of activity, respectively. These low activities make it highly improbable that peptidases play any significant role in egg deterioration, particularly since no known substrate occurs in eggs.

### CATALASE

The occurrence of catalase in hen eggs has been reported by a number of investigators, although Koga (7) failed to find it by his method. Pennington and Robertson (5) observed about a 10-fold variation in catalase content from egg to egg. They also observed that the white contained much more catalase than the yolk.

### *Method*

Catalase was determined by measuring the rate of oxygen liberation from the reaction mixture in the Warburg apparatus at 30°C. The reaction mixture contained 0.5 ml. of 16% dextrose, which stabilizes catalase; 0.2 ml. of 1 *M* pH 7.0 phosphate buffer; the egg sample (1-2 ml.) previously adjusted to pH 7.0 with either 0.5 *N* hydrochloric acid or 0.5 *N* sodium hydroxide; 0.16 ml. of 0.1 *N* hydrogen peroxide; and sufficient distilled water to give a total volume of 3.8 ml. The well of the Warburg vessel contained 0.2 ml. of 10% potassium hydroxide. No preservative was used, since the experiments were of short duration.

The first-order *k* values ( $\log_{10}$ , min.<sup>-1</sup>) were computed from several readings of the oxygen evolved over a 15-minute period. The "*Katalase Fähigkeit*" or *Kat. F.*, defined in this case at 30°C., is obtained by dividing *k* by the g. of enzyme (egg) equivalent to 50 ml. of reaction mixture. The activity in terms of microequivalents of hydrogen

peroxide decomposed/minute/g., is 2.3 times *Kat. F.* times microequivalents/50 ml. of reaction mixture, or, for the concentration of hydrogen peroxide used (0.0042*N*) it is 2.3 (*Kat. F.*) (0.0042  $\times$  50  $\times$  1000) or 483 (*Kat. F.*), or  $k \times 483 \times 3.8/(50 \times \text{g. dry weight per vessel})$ , or  $k \times 37/(\text{g. dry weight per vessel})$ .

### Results and Discussion

Our results confirm earlier observations (Table VIII). The variations in enzyme activity from egg to egg were not due to microbial contamination (see "General Consideration of Methods"). From our limited data it appears that about 85% of the catalase of the whole

TABLE VIII  
*Catalase Activity of Eggs and Its Inactivation by Heat and at pH 3*

Eggs used in test	Catalase activity, <i>Kat. F.</i> $\times 1000$		
	Whole egg <sup>2</sup>	Yolk <sup>2</sup>	White <sup>2</sup>
Storage eggs	$\left\{ \begin{array}{l} 5.8 \\ 1.0 \\ 6.6 \\ 1.8 \end{array} \right.$		
4-Day-old eggs	2.5	0.4	7 <sup>3</sup>
5-Day-old eggs			$\left\{ \begin{array}{l} 58 \text{ Tn} \\ 69 \text{ Tk} \end{array} \right.$
8-Day-old eggs	7.9	1.0	15
9-Day-old eggs			36
11-Day-old eggs			57
17-Day-old eggs			42
32-Day-old eggs	4.6		
40-Day-old eggs			39
57-Day-old eggs			31
11-Day-old eggs held at pH 3 and 25°C.: for 3 hrs.			1.4
for 6 hrs.			0.3
32-Day-old eggs heated to 63°C. for 1-2/3 hrs.	0.2		

<sup>1</sup> The *Kat. F.* reported here is for 30°C. rather than the usual 0°C.

<sup>2</sup> It has been assumed that whole egg has 27% solids; yolk 51% solids; and white 14% solids.

<sup>3</sup> A different batch of 4 day-old eggs gave a *Kat. F.* of  $49.6 \times 10^{-3}$ .

eggs is in the white, and that, on an equal volume basis, the white has about 5 times the catalase activity of the yolk. Pennington and Robertson (5) showed that the catalase activity of hen eggs was destroyed by boiling, and therefore was due to a heat-labile factor. The data in Table VIII further show that catalase is about 95% destroyed by heating the whole liquid egg to as low a temperature as 63°C., which does not cause coagulation. The enzyme of the white is largely destroyed by holding the white at pH 3 and 25°C. for several hours. This is in accord with the acid instability of catalases obtained from other sources (35, p. 172). It is interesting to note that, on the assumption that the *Kat. F.* of pure egg catalase is 40,000 at 0°C., which is about the value found by Sumner for crystalline horse liver catalase (35, p. 173), then the catalase in egg white must be less than 0.0001% of the total solids.

### Summary

The catalase content of eggs, most of which is in the white, is small in comparison to that of many biological materials, and varies about 10-fold from egg to egg.

### OXIDASE

Herlitzka (36) in 1907 reported the presence of an indophenol oxidase in the yolk of hen eggs. Koga (7) in 1923 failed to confirm Herlitzka's results on yolk, but reported that the whites of unincubated eggs contained an oxidase that catalyzed the oxidation of catechol, adrenalin, and dihydroxyphenylalanine, but not tyrosine. The reports of these qualitative results, considering the methods that were available and used then, are not very convincing. For one thing, the work was done before the importance of controlling the pH was fully appreciated.

### Method

Catechol, hydroquinone, and *p*-phenylenediamine were used as substrates in the oxidase studies. Koga's (7) qualitative work, based on observations of color change, has been repeated by us exactly as he described it. In addition, quantitative studies were made in which the rate of oxygen uptake was measured in the Warburg apparatus at 30°C. The shaking rate, 100 excursions per minute, was found to be adequate. The complete reaction mixture used in the quantitative work consisted of egg fraction (0.5–2.0 ml.) adjusted to the desired pH value; phosphate or veronal buffer of the required pH (final concentrations 0.01–0.1 *M*); substrate (final concentrations 0.1–0.2%); and water to give a total volume of 4.0 ml. The substrate solutions were placed initially in the arms of the vessels and were tipped into the main portions of the vessels after thermal equilibrium was achieved. The initial and

final pH values of the contents of all vessels were recorded. Results were considered final only when the desired pH values were maintained.

In addition to measuring the rates of oxidation by observing the rates of oxygen uptake, the rates of color formation were determined on 40 ml. of the complete reaction mixture, and on this mixture less the egg white. These mixtures were maintained at 30°C. in a water bath with continuous aeration with CO<sub>2</sub>-free air. The electrodes of the pH meter were immersed in the solutions and a constant pH value was maintained. At desired intervals, approximately 4 ml. portions of these mixtures were quickly removed, the color content evaluated in a Klett-Summerson colorimeter (Filter No. 42, which transmits light near 420 m $\mu$ ) and the aliquots returned to the reaction mixtures. By this means the rate of increase in color intensity was followed.

### *Results and Discussion*

The observations on which Koga based his conclusions that egg white contained an oxidase have been confirmed with catechol as the substrate. Catechol is oxidized aerobically at the pH of egg white at an appreciable rate; hence, on addition to egg white, aerobic oxidation occurred as a result of the favorable pH of this medium. The oxidation appeared to be enzymic, since a similar solution of egg white heated in a boiling water bath for 30–45 seconds remained colorless for some time after the addition of catechol. This failure to obtain colored oxidation products at a rate comparable with the rate observed at the same pH value, 8.5, in the absence of egg white might have been due to the "release" of sufficient --SH or other reducing groups by heat denaturation of the egg white proteins to continually reduce the oxidation product of catechol back to catechol. Such behavior would be similar to the behavior of native and denatured egg albumin toward ferricyanide and nitroprusside. Native egg albumin does not reduce ferricyanide or nitroprusside, whereas denatured egg albumin reduces both reagents readily (37). This characteristic of egg albumin was, of course, not known at the time of Koga's work.

If the release of reducing groups by heat treatment caused Koga's observations, the catechol alone, catechol plus raw egg white, and catechol plus heated egg white should all exhibit about the same rate of oxygen uptake. Experiments carried out in the Warburg apparatus showed that the rates of oxygen uptake for catechol alone and for catechol plus raw egg white were similar, but for catechol plus heated egg white the rate was much lower (Fig. 3). This lower rate, however, was not maintained. Catecholase did not appear to be present, since the raw egg white did not accelerate the oxygen uptake by the buffered

catechol. Apparently, denatured egg white inhibits the chemical oxidation of catechol. Tests with an amount of crystalline egg albumin equivalent to the egg white used in the other tests showed that native egg albumin had practically no effect, but denatured egg albumin caused an even stronger inhibition of catechol oxidation than did denatured egg white (Fig. 3). Nitroprusside tests for  $-SH$  were negative for native egg white and egg albumin and were positive for the denatured systems. With the latter systems the test became negative again when the inhibition phase shown in Fig. 3 was over. This oc-

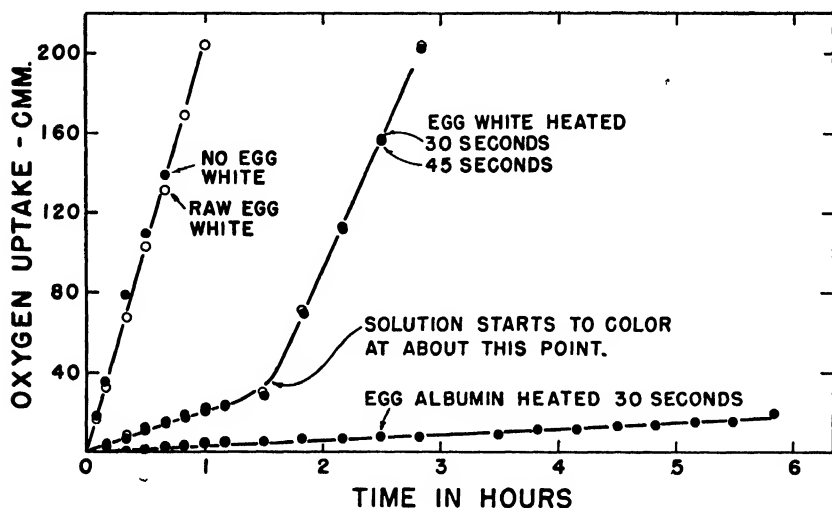


FIG. 3. Oxidation of catechol at pH 8.50 in the absence and in the presence of raw and heat-treated egg white, and in the presence of denatured crystalline egg albumin.

curred at about the same time that the solutions began to show the brown quinone color. In experiments with cysteine, no inhibition effect, such as was obtained with egg albumin, was observed. These results show that Koga's "positive" results were due to production of substances or reactive groups by heat denaturation that inhibited the nonenzymic oxidation of catechol, rather than to enzyme inactivation. It is noteworthy that Koga observed what he considered to be oxidase activity on substrates that are subject to aerobic oxidation at the pH of egg white but not on tyrosine, which is relatively stable at this pH in the presence of oxygen. Table IX shows that various egg fractions

TABLE IX

*Substrates, pH Values, and Egg Fractions Used in Egg Oxidase Studies*

Substrate	pH value	Egg fraction		
		Yolk	White	Whole egg
Catechol	6.1	*	*	
	7.2	*		
	8.5	*	*	
Hydroquinone	6.1	*	*	
	6.5	*		
	7.2	*		
	7.5			*
<i>p</i> -Phenylenediamine	6.7	*		
	7.2		*	
	7.6	*		*

\* No activity within experimental error of method, hence less than 0.0003 unit/kg. dry weight.

did not accelerate the rate of nonenzymic oxidation of catechol, hydroquinone, or *p*-phenylenediamine. In the case of catechol and hydroquinone it was necessary, as previously stated, to control the pH very accurately to correct for nonenzymic oxidation of these substrates at the higher pH values.

Even though the egg white did not accelerate the rate of oxidation of catechol as judged by the oxygen consumed, the rate of color formation in the presence of egg white was greater than in its absence (Table X). The rate of color formation at pH 8.5 was a function of the

TABLE X

*Rate of Color Formation from Catechol at pH 8.5 in the Presence and Absence of Egg White, as Measured in a Klett-Summerson Colorimeter*

Aeration rate	$\Delta$ Color increase per hour		
	With egg white	Without egg white	Net
<i>bubbles/minute</i>			
120	150	50	100
600	298	106	192

rate of aeration, both with and without egg white. A 5-fold increase in aeration rate doubled the rate of color formation for both solutions and, hence, also the net values. Excess foaming prevented use of more vigorous aeration. At lower pH values the slower rate of color formation, together with the tendency for the color to fade made quantitative work unfeasible. The greater rate of color formation in the presence of egg white, considered by itself, would suggest that the egg white was catalyzing the oxidation of catechol. Considering the fact that the oxygen uptakes are the same in the presence and absence of egg white makes this position untenable, however, and illustrates the well known fact that colorimetric methods may be very misleading under certain conditions. Although the reason for the greater rate of color formation in the presence of egg white is not known, one postulate would be that the quinone resulting from the oxidation of the catechol may combine with a constituent of egg white in some manner to produce a more intense color. If such is the case, it appears that the egg white must be present at the time the catechol is oxidized, for addition of egg white to oxidized catechol did not result in immediate intensification of color.

### *Summary*

The observations that led Koga to report the presence of an oxidase in egg white have been repeated and confirmed. However, additional work incorporating essential controls has shown that egg white does not contain polyphenol oxidase activity.

### CYTOCHROME OXIDASE

The important role cytochrome oxidase plays in the oxidative system of animal tissues would suggest that this enzyme might occur in hen eggs. Allen (38), in 1940, reported cytochrome oxidase in developing grasshopper eggs, but was unable to detect it in the freshly laid eggs. In the present studies, in addition to testing for the oxidase, hen eggs were also tested for cytochrome c and for a reduction system capable of reducing oxidized cytochrome c.

### *Method*

The rate of oxygen uptake was measured in the Warburg apparatus at 30°C. for systems that were complete in all the necessary factors except the one for which the

tests were made. Verification of the adequacy of test conditions was always made by adding a known source of the substance in question.

Cytochrome c was prepared according to Keilin and Hartree (39), and cytochrome oxidase by the method of Haas (40, Expt. II, page 483). These substances, together with hydroquinone, or *p*-phenylenediamine, were used in testing egg yolk and egg white for cytochrome oxidase, cytochrome c, and a reducing system, capable of reducing oxidized cytochrome c. All the tests were made at pH 7.2 in the presence of 0.075 *M* phosphate buffer.

### *Results and Discussion*

Within the experimental error of the method (0.0003 unit/kg. of dry weight) egg white and egg yolk contain no cytochrome oxidase activity. Furthermore, no cytochrome c, or reducing system capable of reducing oxidized cytochrome c, could be detected.

The lack of cytochrome oxidase and its substrate cytochrome c in hen eggs, together with the absence of polyphenol oxidases, argues against enzyme involvement in the observed oxygen uptake of dried egg powder (41). Earlier observations (14) that whole fresh eggs do not respire are not surprising, in view of the apparent complete lack of a respiratory system in unincubated fresh eggs.

### *Summary*

Neither the white nor the yolk of hen eggs contains any cytochrome oxidase, cytochrome c, or reducing system capable of reducing oxidized cytochrome c.

### PEROXIDASE

Tests with white and with yolk separately were negative in all cases. These results were not due to reducing substances in the egg which would prevent accumulation of the colored oxidation product. The peroxidase activity was estimated to be zero within the limit of the method, which was about  $\pm 0.01$  unit/kg. dry weight. Engelhardt and Vaehner (43) reported that peroxidase was absent in fresh eggs but appeared in the embryo simultaneously with the appearance of hemoglobin.

Guaiacol and benzidine were used as the substrates in the tests for peroxidase. The concentrations of these substrates and of hydrogen peroxide were such as to give close to the maximum sensitivity (42). The reaction mixtures were buffered at pH 5.6 with 0.05 *M* acetate buffer and observed for color changes for 24 hours.



## AMYLASE

Several investigators have studied the amylase of eggs, since Müller and Masayama in 1899 and 1900 found that it occurs in the yolk of unincubated hen eggs (see Refs. 2 and 3 for general literature). The enzyme was shown to be an  $\alpha$ -amylase similar to salivary amylase (7) and to exhibit optimum activity at about pH 7 (44). The amylase content of the yolk, which varied about 4-fold, was found to be about 30 times that of the white (7).

*Method*

The 100 ml. of reaction mixture consisted of 0.5% starch, 0.04 *M* phosphate buffer (pH 6.8), 0.5 *M* NaCl, and 4 ml. of yolk or 0.3 g. of livetin. The increase in reducing groups at 37°C. was determined by the modified Willstätter-Schudel hypiodite method (45). The activity was based on the results obtained in a few hours; hence no preservative was used. The rate of hydrolysis was constant until about 10% of the total glucoside bonds were hydrolyzed, but was markedly lower after 15% hydrolysis.

*Results and Discussion*

The data reported here demonstrate that egg yolk amylase appears in the livetin fraction of the yolk (Table XI). On the basis of the

TABLE XI

*Amylase Activity of Egg Yolk and Livetin*

Enzyme source <sup>1</sup>	Units/kg. dry weight
Yolk sample 1	1.7
Yolk sample 2	1.4
Livetin	6.2

<sup>1</sup> Each yolk sample was a mixture of the yolk of 3 eggs.

activities reported in Table XI only about 25% of the total yolk amylase appeared in the livetin fraction. However, the livetin was not prepared from the yolk samples that were assayed directly; therefore, the low yield might have been merely a reflection of differences in enzyme content, which Koga found to vary.

## PROTEINASE

The presence of a proteinase in egg white has been a controversial issue.

Balls and Swenson (13) reported a tryptic proteinase in thick egg white and considered the enzymic alteration of its mucin content to be responsible for the change from thick to thin white. Their report was challenged by Van Manen and Rimington (8), who were unable to detect proteinase activity in egg white, although they reported erepsin to be present (*cf.* section on erepsin). Balls and Hoover (46) concluded, after a very detailed study, that "there is little or no active proteinase present" in egg white. Thompson (11), using casein as a substrate, was unable to detect any significant amount of proteolysis, but reported increases in tyrosine content of filtrates prepared from experiments in which "strands of gut" were used as substrate. Schultze (9) studied the effect of egg white on peptone, glycylglycine, gelatin, and casein. He reported activity only with peptone and glycylglycine. No proteinase action was detected.

The evidence available at this time fails to substantiate the position that egg white contains proteinase activity. However, since ovomucoid of egg white is a potent trypsin inhibitor (47) considerable trypsin could exist in egg white without being detected.

### GENERAL DISCUSSION

In a study of the reports in the literature involving enzymes in eggs, one is immediately struck with the necessity of evaluating the reliability of the methods that have been used. Some of the original reports that have been referred to most frequently in later writings are not at all convincing in the light of information now available. Lack of adequate control of microbial contamination has been responsible for some reports that are misleading. Misinterpretation of results has led to other false reports; for example, the importance of pII was not fully appreciated at the time Koga (7) reported the presence of oxidases in egg white.

The low order of enzyme activity in eggs has no doubt been largely responsible for the confusing reports that have appeared in the literature. The experiments have usually been of long duration due to the slight activities encountered, and this has made the control of microbial contamination important. Furthermore, the need for adequate control of chemical reactions is much greater in those cases where little or no activity is encountered.

Only relatively few enzymes have been found in eggs and none occurs in large amounts. Table XII contains a summary of the work reported here. As methods are refined, no doubt other enzymes will be found in small quantities. The low enzyme content of hen eggs is consistent with observations on dormant seeds, which also are pre-

TABLE XII

*Summary of Egg-Enzyme Activities*

Enzyme	Units/kg. dry weight		
	Yolk	White	Livetin
Amylase	1.50		6.00
Tributyrylase	0.40	0.10	5.00
Peptidase	0.10	0.15	
Phosphatase	0.50	<0.02 <sup>1</sup>	
Catalase	0.70	50 <sup>2</sup>	
Lipase <sup>1</sup>	<0.002		<0.02
Oxidase <sup>1</sup>	<0.0003	<0.0003	
Cytochrome oxidase <sup>1</sup>	<0.0003	<0.0003	
Peroxidase <sup>1</sup>	<0.010	<0.010	

<sup>1</sup> Absent within the experimental error of the method used.

<sup>2</sup> The units for catalase are not comparable with the other units, which, however, are comparable with each other (see text).

cursors of embryonically active tissues. It is reasonable to postulate that developing embryos control the enzyme activity of the egg or seed and that the fresh egg or dormant seed would, therefore, possess little enzyme activity.

The recognized low enzyme content of eggs, as well as the lack of recognized substrates for some of them, lend but little support to the belief that deterioration of processed eggs, frozen or dried, is caused by the inherent enzymes. There may be instances, however, where enzymes resulting from contaminating organisms are responsible for changes in egg substance (48). These changes may, or may not, lead to loss in quality, depending on the types of organisms present and the circumstances.

## SUMMARY

Reinvestigations of enzymes that have been reported to occur in hen eggs have confirmed some of the earlier reports but failed to confirm others. Some enzymes have been erroneously reported to be present, as a result of faulty procedures. Autolipolysis of egg yolk, reported to occur by Koga and Wohlgemuth, has been shown to occur only in the presence of contaminating microorganisms. Tributyrinase occurs in both the yolk and white, but is present in much larger quan-

tity in the yolk and is quantitatively associated with the livetin fraction of the yolk. It is completely inhibited by eserine. Esterases capable of hydrolyzing methylbutyrate, benzylbutyrate, acetylcholine, benzoylcholine, acetyl- $\beta$ -methylcholine, triacetin, and tripropionin, in addition to tributyrin, were found in the livetin fraction of yolk. Amylase occurs in the yolk and is, at least partially, associated with the livetin fraction. Small amounts of peptidase activity (crepsin) appear in both the yolk and white. Measurable amounts of phosphatase were found only in the yolk. The white contained most of the egg catalase; however, the yolk contained significant amounts.

Lipase, active on lipides containing fatty acids with 6 or more carbons, phenol oxidase, cytochrome oxidase, and peroxidase could not be detected by the methods used.

The activities, while variable from enzyme to enzyme, are so low (less than two milliequivalents of substrate bonds split/kg. egg/minute at 30°C.) that it appears extremely doubtful that egg enzymes are primary causes of egg deterioration.

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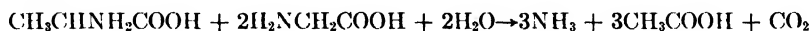
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## LETTERS TO THE EDITORS

### Extension of the Stickland Reaction to Several Bacterial Species

Sirs:

Stickland (1, 2) has shown that a peculiar type of reaction between amino acids occurs in the presence of cell suspensions of a strict anaerobe, *Cl. sporogenes*, one amino acid acting as hydrogen donor, the other as hydrogen acceptor, both being deaminated during the reaction. Between alanine and glycine, the reaction can be written as follows:



Clifton (3) found that this reaction also occurs with *Cl. botulinum*. We were able to find that the following species can carry out a Stickland reaction between alanine and glycine, as well as that between alanine and proline: *Cl. histolyticum*, *Cl. flabelliferum*, *Cl. saprotoxicum*, *Cl. sordellii*, *Cl. bifermentans*, *Cl. butyricum*, *Cl. acetobutylicum*, *Inflabilis indolicus*. All these species, with the exception of *Cl. butyricum*, are proteolytic.

The following species were not able to carry out such a reaction: *Cl. iodophilum*, *Cl. saccharobutyricum*, *Plectridium tetanomorphum*, *Welchia perfringens*, *Inflabilis teras*, *Plectridium tetani*. With the last species, we obtain the same results as Clifton (4). All these bacteria are non-proteolytic.

The following facultative anaerobes do not carry out a Stickland reaction: *Staphylococcus aureus*, *Proteus vulgaris*, *Klebsiella pneumoniae*, and *Escherichia coli* (5).

All the species we described as giving the Stickland reaction may carry it out with pyruvate, acetaldehyde or ethanol, but not with lactate or formate, as hydrogen donors.

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## The Preparation of Apyrase from Potato

Sirs:

The name "apyrase" has been given to the enzyme which splits off two phosphate groups from ATP, as contrasted with ATP-ase, which splits off only the terminal phosphate group. Enough evidence has not yet been forthcoming to establish whether apyrase is a specific, individual enzyme.

The best known apyrase is that from potato, described first by Kalckar (1). His procedure consisted in preparing an aqueous extract of potato, freeing this from certain proteins by addition of ammonium sulphate to about 0.6 saturation and precipitating the enzyme by evaporating the supernatant to complete saturation with respect to ammonium sulphate. The precipitate was collected on filter paper, redissolved in water and dialyzed against water. A solution was obtained which was strongly pigmented and which could be further purified by fractionation with ammonium sulphate, or adsorption on aluminum hydroxide, followed by elution and fractionation with alcohol. The purest product obtained split off 9  $\gamma$  of P/ $\gamma$  of protein. The time interval in the standard test was 5 minutes and no data were reported as to whether, with a longer period of action, the preparation would split more than two phosphate groups from ATP.

Meyerhof (2) effected slight modifications in Kalckar's method. Inert proteins were precipitated from the extract by 0.4 saturation with ammonium sulphate, instead of 0.6 saturation. The filtrate was then taken to full saturation, the precipitated enzyme collected and dissolved in water, and dialyzed. A solution was obtained which was only 10% as active as Kalckar's preparation and which was still contaminated with small amounts of phosphatases.

We have been able to reproduce neither Kalckar's nor Meyerhof's observations. Our extracts were made by grinding sliced potato in the Waring blender for 2-3 minutes with ice cold neutralized *M*/100 potassium cyanide. In this way we have

avoided the pigmentation of the enzyme fractions. The observations recorded below hold good also when the extracting medium does not contain cyanide and is made up of water only, or  $M/2$  potassium chloride. We have also considered it advisable to allow a longer period of action of the enzyme, namely, 30 minutes, to make certain that the preparation is free from the presence of contaminating phosphatases. In direct contrast to the observations of Kalekar, we find that practically the entire ATP-splitting activity of the extract is concentrated in the solid fraction obtained by 0.6 saturation with ammonium sulphate, as is evident from the accompanying table. With Meyerhof's modified procedure, appreciable quantities of the activity were retained in the precipitate by 0.4 saturation (*vide* table below).

*Fractionation of Potato Extract with Ammonium Sulfate*

0.6 Saturation Per cent of original activity	Full saturation of filtrate Per cent of original activity
138	3
123	2
0.4 Saturation	Full saturation of filtrate
49	96
41	82
32	90

(In both cases the activity recovered after fractionation was in excess of the activity originally present, indicating the possible presence of inhibiting agents in potato extract.)

In our studies on apyrase we have, therefore, precipitated cyanide extracts of potato by 0.6 saturation with ammonium sulfate, collected the precipitate on filter paper, suspended in water and dialyzed overnight against distilled water in the cold room. The material was centrifuged and the supernatant and the residue, both of which were enzymically active, worked up separately. At this stage both the fractions were very crude, since 95–96% of the total P of ATP was split off. The supernatant solution, which contained the larger part of the activity, was then reprecipitated by 0.6 saturation with ammonium sulphate, taken up in water and dialyzed. A solution was obtained which split 81% of the total P of ATP and which had a purity representing some 50-fold concentration of the original activity of dried potato; the actual amount of P split was 900–1100  $\gamma$  P/mg. dry weight of solid, a period of 30 minutes being allowed for the action of the enzyme in the standard test. In one experiment a simple fractionation of such a solution with ammonium sulphate yielded a preparation which split over 60,000  $\gamma$  P/mg. dry solid, corresponding to over



2700-fold purification. We have, therefore, reason to believe that a major part of the ATP-splitting enzyme of potato behaves as an albumin in solubility properties, but as a globulin with respect to precipitation by ammonium sulphate in the neighborhood of half saturation.

#### ACKNOWLEDGMENTS

The author's thanks are due to Professor James B. Sumner, without whose encouragement this investigation would not have been possible, to the Rockefeller Foundation for financial aid and to Cornell University for the award of a Fellowship.

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## Book Reviews

**Annual Review of Biochemistry. Vol. XVI.** Edited by J. M. LUCK, H. S. LORING and G. MACKINNEY. Annual Reviews, Inc., Stanford, California, 1947. 740 pp. Price \$6.00.

The appearance of a volume of the *Annual Review* is always welcomed by the present writer. For the past 13 years the University of Cambridge has held courses in fundamental biochemistry, both elementary and advanced, which are certainly unique, at least in Europe, if not further afield. In these courses, comparative biochemistry in the widest sense is taught as an individual scientific discipline, and is independent of the course of chemical physiology where emphasis is laid on the chemical events associated with human physiology alone. Without some such aid as that provided by the *Annual Review* the tasks of the writer and his colleagues would indeed be grim. Some idea of the vastness of the literature of biochemistry is afforded by the fact that the present volume reviews the work of over four thousand investigators. There are likely to be quite a number of papers which are not referred to, during the period under consideration.

The research worker, as well as the teacher, finds the *Annual Review* of great value, particularly in the study of those fields which abut on that which is one's especial interest. It is, therefore, of the utmost importance that the author of a review should adopt a critical attitude in his reporting and not submit a mere card-index, re-constituted somewhat in the manner of our so-called "Household Milk" which, for the benefit of the American reader, is dried skim-milk and is an important source of dietary protein in the U. K. The writer, having tried to indicate his feelings toward the *Annual Review* in general, has now the duty of expressing his opinion of Volume XVI in particular. It so happens that he reviewed Volume XV, a year ago, in a British periodical. He then made certain generalized criticisms, mainly on the lines that the contributors to Vol. XV had not received sufficient editorial direction regarding the subject matter of their review. As a result, much space was wasted by quite unnecessary repetition and the time of the contributors had likewise been wasted. Of all people, the scientist is one who has so much to do and too little time in which to do it. This year, the writer is pleased to find, the *Annual Review* contains little or no overlap between its articles and the critical outlook is everywhere evident. While analyzing the contents of Volume XVI, the writer was struck by the fact that, in addition to a complete article on "Pteroylglutamic Acid in Clinical Studies" (12 pp. and 32 refs.), the folic acid-pteroylglutamic acid question was a feature of "Water-Soluble Vitamins" where it occupied 4 pp. and had 36 references. However, only about 6 of these references overlap. It is to be hoped that future *Annual Reviews* will continue to be as well edited.

As we have become accustomed to expect, Volume XVI begins with "Biological Oxidations and Reductions." Why this lengthy title? We are always taught that every

oxidation is accompanied by a reduction! [Incidentally the second paragraph of this article contains the only error which forcibly obtruded itself on the writer, namely "l-malonate" for l-malate]. This subject continues to attract much interest, the most novel item reported is the growing realization of the part played by ATP in facilitating so many metabolic processes. This article, rather oddly in view of its title, contains a page devoted to hexokinase.

The review on "Proteolytic Enzymes" is a straightforward account of progress along recognized lines, while "The Chemistry of the Carbohydrates" deals specially with chemical aspects of amino sugars, uronic acids, sugar anhydrides (including their use in preparing rare sugars), unusual reactions (including the first synthesis of crystalline L-fructose), disaccharides, polysaccharides of plant gums and algae (including useful summaries of present structural knowledge), and a final section on some diverse analytical methods. In "The Chemistry and Metabolism of the Lipides" we find an adequate review of progress in this notoriously refractory field. Herein is recorded an interesting fact regarding the antagonism of C<sub>7</sub>, C<sub>8</sub>, C<sub>11</sub>, and C<sub>13</sub> fatty acids toward the ringworm fungus. These acids are said to occur in the hair follicles of adults, but not of children, thus explaining why this juvenile affection clears up with the onset of adolescence. "Phosphorus Compounds" deals largely with analytical approaches and the widespread biological employment of ATP, while "Carbohydrate Metabolism" is largely concerned with summarizing the influence, in the animal, of various intrinsic and extrinsic factors including a section on the so-called "Alloxan Diabetes." 235 papers are considered.

The contribution on "Metabolism of Protein," written by a nutritionist, is of especial value, as it has a somewhat "applied" twist. In conjunction with the unusually instructive and critical article on the "Chemistry of Proteins and Amino Acids," this field is well provided for.

A number of rarely reviewed subjects are included this year. All are of interest and the critical aspect is, on the whole, well maintained. "Anti-oxidants," "Choline," "Pteroylglutamic Acid in Clinical Studies," "Animal Pigments," "Growth Substances in Higher Plants," "Marine Bacteriology," "Anti-malarial Drugs" and "Use of Isotopes in Biochemical Research" are the titles. The last mentioned is a most informative and critical study, indicating practical difficulties and pointing to possible future developments.

Among the remaining articles, which are of the "hardy annual type," we find the critical spirit pervading throughout. This is ever necessary among all scientists, and perhaps among biochemists in particular.

D. J. BELL, Cambridge, England

**Actualités Biochimiques, Conception Actuelle du Catabolisme de l'Hémoglobine.** By CLAUDE LIÉBECQ, Laboratory of Biochemistry, University of Liège. Masson et Cie., Paris, 1946. 63 pages, price 140 fr.


Although the composition and structure of the hemoglobin molecule are perhaps better known than any other protein, and the chemistry of the bile pigments has become better established during recent years, the pathways of hemoglobin decomposition to the bile pigment level are still in a controversial stage. This results from the fact that the immediate breakdown products of hemoglobin are a complex mixture

which cannot be separated satisfactorily because they differ little in their solubilities. They have, therefore, been characterized primarily by the changes in position of several of the visible absorption bands, often only one band in the red being considered for study. Various compounds such as ascorbic acid, phenylhydrazine, sulfanilamides, hydrogen sulfide, *etc.*, bring about an irreversible change in absorption. A number of these compounds appear, indirectly, to bring about an oxidation of hemoglobin, giving rise to various combinations of oxidized hemes and oxidized and denatured globins which are difficult to disentangle on the basis of spectroscopy alone. (Characterization of some of these intermediate materials by combining known oxidation products of heme with known derivatives of globin has yet to be attempted.)

After a brief introduction into the formation of bilirubin and the other bile pigments, Liébecq reviews in some detail the studies of hemoglobin decomposition by Lemberg, Barkan and their coworkers, including some of the author's own work in this field. The author has undertaken the difficult task of classifying and unifying the complex terminology assigned to these ill-defined substances by various workers. For example, he suggests that the "pseudohemoglobin" of Barkan is probably identical with the "choleoglobin" of Lemberg.

This review by Liébecq will be welcome to those interested in the field of hemoglobin catabolism, not only because of its attempt to summarize concisely the present state of our knowledge in this field, but also because it covers European literature of the war years which has only recently become available in this country.

S. GRANICK, New York, N. Y.

 **An Introduction to Bacteriological Chemistry**, 2nd ed. By C. G. ANDERSON. Wellcome Physiological Research Laboratories, Langley Court, Beckenham, Kent, England. Williams and Wilkins Co., Baltimore, 1946. x + 500 pp. Price \$4.00.

The general outline for this edition is the same as the last in that the book is divided into 3 parts: "General Consideration," with 5 chapters; "Metabolism," with 16 chapters; and "Some Aspects of Immunochemistry," with 2 chapters. The chapter headings are: (1) Introduction; (2) Hydrogen Ion Concentration and pH; Oxidation-Reduction Potentials; (3) Colloids and Adsorption; (4) Enzymes; (5) The Chemical Composition of Bacteria, Yeasts and the Lower Fungi; (6) The Nutrition of the Autotrophic Bacteria; (7) The Nutrition of the Heterotrophic Bacteria; (8) Adaptive and Constitutive Enzymes; (9) Growth Factors; (10) Chemotherapy; (11) Antibiotics; (12) Bacterial Respiration; (13) Nitrogen Metabolism; (14) Carbon Metabolism; (15) Alcoholic Fermentation; (16) The Fermentation Products of the Lower Fungi; (17) Industrial Fermentations; (18) The Proteins of Microorganisms; (19) The Polysaccharides of Microorganisms; (20) The Lipoids of Microorganisms; (21) The Pigments of Microorganisms; (22) Antigens, Haptens, Antibodies and Complement; (23) The Mechanism of Antigen-Antibody Reactions. The chapters on chemotherapy and antibiotics are new in this edition, and contain much recent information. The book has a good general index, and two appendices: The Isolation and Identification of Metabolic Products, and Synonyms of Microorganisms.

Although the two new chapters constitute the main difference between the first and the present edition, much new material has been added to each chapter. In fact, the author is to be commended for the way in which he has brought certain chapters

up to date without greatly increasing the size of the book. The subject matter covered is very clearly discussed, and many chemical formulae are used to aid the beginning student, for whom this book is primarily intended. The main criticism leveled against the first edition of the book was that it contained too few references. This point has been partially taken care of in this edition by adding more references at the end of each chapter. However, one must agree with the opinion of the author that a detailed bibliography is out of place in a textbook of this type.

The book is well printed and bound, and is highly recommended for students and others who feel the need for some general understanding of the metabolic activities of microorganisms.

J. R. PORTER, Iowa City, Iowa.

**Les cancers produits par les rayonnements électromagnétiques.** By ANTOINE LACASSAGNE, Professor, Collège de France, and Director, Institut du Radium, Paris Hermann & Cie, Editeurs, Paris, 1945. 138 pp. Price Fr. frs. 150.

The distinguished French scientist presents a comprehensible short revue of the clinical and experimental data collected in the literature concerning the carcinogenic action of radiations. Lacassagne complements them with the results of investigations conducted at the Radium Institute of the Institut Pasteur in Paris. In the particular chapters of the book he discusses (1) the theoretical explanation of the effects of absorption of electromagnetic waves in the body, (2) cancer provoked by X-ray and gamma irradiation, (3) skin cancer produced by sun-ray irradiation, (4) experimental cancer by ultraviolet radiations, (5) skin cancer following burns, (6) cancer appearing in the deep seated body tissues after X-ray or gamma irradiation. The last chapter is especially instructive, collecting the few known experimental and clinical facts concerning this important but as yet controversial problem. A comprehensive bibliography is added to each chapter. It is a useful book to be recommended for everyone interested in the questions discussed.

HENRY K. WACHTEL, New York, New York

## AUTHOR INDEX

### A

- ALBAUM, H. G., AND KLETZKIN, M.  
Adenosine triphosphate from *Drosophila melanogaster*, 333
- ALDERTON, G. See Lineweaver, 311
- ALLISON, J. B. See Chow, 69
- AMES, S. R. See Mallette, 283
- ANDERSON, H. W. See Thornberry, 389
- AU, M. H. See Lansing, 361
- AVERY, A., AND MUNRO, F. L. Chemical and electrophoretic studies of fibrinogen prepared by various methods, 33

### B

- BABCOCK, V. See Zucker, 115
- BAIN, J. A., AND DEUTSCH, H. F. Studies on lactoglobulins, 223
- BARNES, J. M. See Cunningham, 403
- BAUMANN, C. A. See Clayton, 415
- BEAN, R. S. See Lineweaver, 443
- BERGENSTAHL, D. See Tobin, 373
- BIRD, O. D. See Swendseid, 367
- BOURDILLON, J. Crystalline pepsin-resistant protein from skeletal muscle, 61
- BOWLAND, J. P., ENSMINGER, M. E., AND CUNHA, T. J. Need for an unidentified factor or factors for growth, reproduction, and lactation of rats fed purified rations, 257
- BOWMAN, D. E. Further differentiation of bean trypsin-inhibiting factors, 109
- BRAND, J. See Broh-Kahn, 87
- BROH-KAHN, R. H., MIRSKY, I. A., PERISUTTI, G., AND BRAND, J. The hexosemonophosphatase system (glucose-6-phosphatase) of the liver, 87
- BROWN, A. H., AND FRANCK, J. On the participation of carbon dioxide in the photosynthetic activity of illuminated chloroplast suspensions, 55

- BROWN, R. A. See Swendseid, 367
- BURRELL, R. C. See Houston, 299

### C

- CARNELLY, H. L. See Lauffer, 321
- CARRUTHERS, C. See Roberts, 241
- CASCAJARES, M. L. See Giral, 179
- CHELDELIN, V. H. See King, 233
- CHOW, B. F., SEELEY, R. D., ALLISON, J. B., AND COLE, W. H. The effect of repletion on the plasma proteins in the dog measured by electrophoretic analysis, 69
- CHUDZIK, E. B. See Shipley, 301
- CLAYTON, C. C., AND BAUMANN, C. A. Effect of fat and calories on the resistance of mice to 2,4-dinitrotoluene, 415
- COHEN, G. N. See Nisman, 473
- COLE, W. H. See Chow, 69
- CRUESS, W. V., AND SUGIHARA, J. The oxidase of the olive, 39
- CUNHA, T. J. See Bowland, 257
- CUNNINGHAM, L., BARNES, J. M., AND TODD, W. R. Maintenance of carbohydrate stores before and after insulin administration in rats prefed diets containing added glycine, 403

### D

- DAWSON, C. R. See Mallette, 283
- DE LEY, J. See Virtanen, 171
- DEUTSCH, H. F. See Bain, 223
- DISCHE, Z. A new specific color reaction of galacturonic acid, 409
- DUNN, M. S. See Merrifield, 339

### E

- ELLIOTT, R. R. See West, 47
- ENSMINGER, M. E. See Bowland, 257

- ERWAY, W. F. See McShan, 379  
 EVANS, R. J. Sulfur amino acid nutrition of some lactic acid-producing bacteria, 357

## F

- FIGORE, J. V. On the mechanism of enzyme action. XXXII. Fat and sterol in *Fusarium lini* Bolley, *F. lycopersici*, and *F. solani* D<sub>2</sub> purple, 161  
 FISCHER, C. J., AND GREEP, R. O. Studies on activation of purified alkaline phosphatase, 201  
 FONTAINE, T. D. See Ma, 399  
 FRANCK, J. See Brown, 55

## G

- GARZA, H. M. See Pallares, 275  
 GEIGER, W. B. Antibacterial unsaturated ketones and their mode of action, 423  
 GIRAL, F. Mexican turtle oils. IV. *Lepidochelis olivacea* Esch., 193  
     See Giral, J., 183  
 GIRAL, F., AND CASCAJARES, M. L. Mexican turtle oils. I, 179  
 GIRAL, F., AND MARQUEZ, A. Mexican turtle oils. III. *Caretta caretta*, Linn., 189  
 GIRAL, J., GIRAL, F., AND GIRAL, M. L. Mexican turtle oils. II. *Chelone mydas*, Linn., 183  
 GIRAL, M. L. See Giral, J., 183  
 GONÇALVES, J. M., AND GREENSTEIN, J. P. Enzymatic hydrolysis of  $\alpha,\alpha$ -di-(glycylamino) propionic acid, 1  
 GREENSTEIN, J. P. See Gonçalves, 1  
     See Taylor, 19  
 GREEP, R. O. See Fischer, 201  
 GYÖRGY, P. See Shipley, 301

## H

- HEINRICH, R. A. See Swendseid, 367  
 HODSON, A. Z. Inhibitory effect of pure and semipurified proteins on the activity of hog kidney conjugase, 309  
 HOLLAENDER, A. See Taylor, 19  
 HOLLISTER, P. See Zucker, 115

- HOTCHKISS, R. D. A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations, 131  
 HOUSTON, F. G., AND BURRELL, R. C. The isolation of inulin and an unidentified C<sub>20</sub> compound from the roots of *Solidago canadensis* L., 299

## K

- KING, H. H. See MITCHELL, 343  
 KING, T. E., AND CHELDELIN, V. H. Pantothenic acid studies. II. The influence of glutamic acid and pantoic acid on yeast growth, 233  
 KLETZKIN, M. See Albaum, 333  
 KLINE, L. See Lineweaver, 443  
 KLOSE, A. A. See Lineweaver, 311  
 KREHL, W. A. See Mayer, 313  
 KRISHNAN, P. S. Oxygen absorption method for the estimation of coenzyme I, 291  
     The preparation of apyrase from potato, 474

## L

- LANSING, A. I., ROSENTHAL, T. B., AND AU, M. H. Ultrafilterable and non-ultrafilterable calcium in normal, hyperplastic epidermis and squamous cell carcinoma, 361  
 LAUFFER, M. A., CARNELLY, H. L., AND MACDONALD, F. Thermal destruction of influenza A virus infectivity, 321  
 LEINFELDER, P. J. See Robbie, 437  
 LEWIS, S. See Mallette, 283  
 LINEWEAVER, H., KLOSE, A. A., AND ALBERTON, G. An alternate step for the isolation of subtilin, 311  
 LINEWEAVER, H., MORRIS, H. J., KLINE, L., AND BEAN, R. S. Enzymes of fresh hen eggs, 443  
 LOUHIVUORI, A. See Virtanen, 151

## M

- MA, R., AND FONTAINE, T. D. *In vitro* antibiotic activity of crystalline toma-

- tine toward *Candida Albicans*. Antagonistic effect of rutin and quercetin, 399
- MACDONALD, E. See Laufer, 321
- MC SHAN, W. H., ERWAY, W. F., AND MEYER, R. K. Malic dehydrogenase and cytochrome oxidase of lutein and other ovarian tissues during pregnancy and lactation, 379
- MALLETTE, M. F., LEWIS, S., AMES, S. R., NELSON, J. M., AND DAWSON, C. R. The preparation of mushroom tyrosinase, 283
- MARQUEZ, A. See Giral, F., 189
- MAYER, J., AND KREHL, W. A. Scorbatic symptoms in vitamin A-deficient rats, 313
- MERRIFIELD, R. B., AND DUNN, M. S. Microbiological determination of cytosine, uracil and thymine, 339
- MEYER, R. K. See McShan, 379
- MIRSKY, I. A. See Broh-Kahn, 87
- MITCHELL, H. L., SCHRENK, W. G., AND KING, H. H. Effect of high-moisture storage on preservation of carotene in dehydrated alfalfa meal, 343
- MORRIS, H. J. See Lineweaver, 443
- MUNRO, F. L. See Avery, 33

## N

- NELSON, J. M. See Mallette, 283
- NISMAN, B., RAYNAUD, M., AND COHEN, G. N. Extension of the Stickland reaction to several bacterial species, 473

## P

- PALLARES, E. S., AND GARZA, H. M. The structure of the alkaloid of yolo-xochitl, 275
- PERISUTTI, G. See Broh-Kahn, 87
- PERLMAN, D. Desthiobiotin and O-heterobiotin as growth factors for "normal" and "degenerate" strains of *Clostridia*, 79

## R

- RAYNAUD, M. See Nisman, 473
- ROBBIE, W. A., AND LEINFELDER, P. J.

- Cyanide inhibition of retinal respiration in bicarbonate buffer, 437
- ROBERTS, E., AND CARRUTHERS, C. Adenylpyrophosphatase activity in epidermal carcinogenesis in mice, 241
- ROSE, D. The amylase of *Bacillus polymyxa*, 349
- ROSENFELD, W. D. Fatty acid transformations by anaerobic bacteria, 263
- ROSENFELD, R. E., AND TUFT, H. S. Preliminary observations on the adsorption of prothrombin by barium sulfate, 329
- ROSENTHAL, T. B. See Lansing, 361

## S

- SCHRENK, W. G. See Mitchell, 343
- SEELEY, R. D. See Chow, 69
- SHIPLEY, R. A., CHUDZIK, E. B., AND GÖRGY, P. The effect of extirpation of various endocrine glands on the production of fatty liver, 301
- STEFFEE, C. H. See Tobin, 373
- SUGIHARA, J. See Cruess, 39
- SWENDSEID, M. E., BROWN, R. A., BIRD, O. D., AND HEINRICH, R. A. Comparative study of pteroylglutamic acid and its hexaglutamyl conjugate in the correction of sulfasuxidine-induced leucopenia in the rat, 367

## T

- TAYLOR, B., GREENSTEIN, J. P., AND HOLLAENDER, A. Effects of X-radiation on sodium thymus nucleate, 19
- THORNBERRY, H. H., AND ANDERSON, H. W. Synthetic medium for *Streptomyces griseus* and the production of streptomycin, 389
- TOBIN, J. R., JR., BERGENSTAHL, D., AND STEFFEE, C. H. The relationship of protein reserves to the production of hyaluronidase and antihyaluronidase, 373
- TODD, W. R. See Cunningham, 403
- TUFT, H. S. See Rosenfield, 329



## V

- VIRTANEN, A. I., AND DE LEY, J. The enzyme activity and nitrogen content of bacterial cells, 171  
VIRTANEN, A. I., AND LOUHIVUORI, A. Estimation of L-aspartic acid with aspartase, 151

## W

- WEST, H. D., AND ELLIOTT, R. R. The role of pantothenate in sulfapyridine-induced achromotrichia, 47

- WOLF, F. T. The amino acid metabolism of *Penicillium chrysogenum* Q-176, 143

## Z

- ZIMMERBERG, H. Y. The metabolism of various phthalein dyes by rat liver slices, 197  
ZUCKER, L. M., ZUCKER, T. F., BABCOCK, V., AND HOLLISTER, P. Zopherin: Nutritional factor for rats associated with animal protein sources, 115  
ZUCKER, T. F. See Zucker, L. M., 115

## SUBJECT INDEX

### A

- Achromotrichia, sulfapyridine induced — and pantothenate, WEST AND ELLIOTT, 47
- Adenosine triphosphate, — from *Drosophila melanogaster*, ALBAUM AND KLETZKIN, 333
- Adenyl pyrophosphatase, — activity in carcinogenesis, ROBERTS AND CARRUTHERS, 239
- Alfalfa, high-moisture storage and preservation of carotene in dehydrated alfalfa meal, MITCHELL, SCHRENK, AND KING, 343
- Alkaloid, structure of the — of Yolochochitl, SODI PALLARES AND GARZA, 275
- Amino acids (see also *Glutamic acid*); — — metabolism of *Penicillium chrysogenum*, WOLF, 143; sulfur — — nutrition of lactic acid producing bacteria, EVANS, 357; Stickland reaction and several bacterial species, NISMAN, RAYNAUD, AND COHEN, 473
- Amylase, — of *Bacillus polymyxa*, ROSE, 349
- Anaerobes, see *Bacteria*
- Analysis (see also *Aspartic acid*, *Coenzyme I*, *Electrophoresis*, *Lactoglobulins*, *Microchemistry*); microbiological determination of cytosine, uracil and thymine, MERRIFIELD AND DUNN, 339; color reaction of galacturonic acid, DISCHE, 409
- Animal, see the individual animals (for instance *Rat*)
- Animal protein factor, see *Zoopherin*
- Antibiotic, see *Subtilin*
- Antihyaluronidase, see *Hyaluronidase*
- Apyrase, preparation from potato, KRISHNAN, 474

- Aspartase, estimation of L-aspartic acid by —, VIRTANEN AND LOUHIUVUORI, 151
- L-Aspartic acid, estimation, VIRTANEN AND LOUHIUVUORI, 151
- Aztequine, SODI PALLARES AND GARZA, 275

### B

- Bacillus polymyxa*, amylase of — —, ROSE, 349
- Bacteria, enzyme activity and nitrogen content of bacterial cells, VIRTANEN AND DELEY, 169; fatty acid transformations by anaerobic —, ROSENFELD, 263; microbiological determination of cytosine, uracil and thymine, MERRIFIELD AND DUNN, 339; sulfur amino acid nutrition of lactic acid producing —, EVANS, 357; antibacterial activity of unsaturated ketones, GEIGER, 423; extension of the Stickland reaction to several bacterial species, NISMAN, RAYNAUD, AND COHEN, 473
- Barium sulfate, adsorption of prothrombin by —, ROSENFELD AND TUFT, 329
- Bean, differentiation of — trypsin inhibiting factors, BOWMAN, 109
- Beef peptomyosin, crystalline — —, BOURDILLON, 61
- Biotin, desthio- and O-hetero- — as growth factors for clostridia, PERLMAN, 79
- Blood (see also *Fibrinogen*, *Leucopenia*); destruction of red — cell-agglutinating activity of influenza A virus, LAUFFER, CARNELLY, AND MACDONALD, 321; prothrombin adsorption by barium sulfate, ROSENFELD AND TUFT, 329

Bovine lactoglobulin, BAIN AND DEUTSCH, 221

## C

Calcium, see *Ultrafiltration*

Calories, see *2,4-Dinitrotoluene*

Cancer, see *Carcinoma*

*Candida albicans*, — — and tomatine, MA AND FONTAINE, 399

Carbohydrates (see also *Microchemistry*); — stores in rats, and insulin and glycine, CUNNINGHAM, BARNES, AND TODD, 403

Carbon dioxide, — — and the photosynthetic activity of illuminated chloroplast suspensions, BROWN AND FRANCK, 55

Carcinogenesis, adenylypyrophosphatase and —, ROBERTS AND CARRUTHERS, 239

Carcinoma, calcium in —, LANSING, ROSENTHAL, AND AU, 361

Caretta, mexican turtle oils (III), — caretta, Linn., GIRAL AND MARQUEZ, 187

Carotene, preservation in dehydrated alfalfa meal, MITCHELL, SCHRENK, AND KING, 343

Catalase, see *Enzymes*

Catecholase, — from mushroom, MALLETTE, LEWIS, AMES, NELSON, AND DAWSON, 283

Cells, see *Bacteria*, *Carcinogenesis*, *Enzymes*, *Ultrafiltration*

Chelone mydas, Mexican turtle oils (II), GIRAL, GIRAL, AND GIRAL, 181

Chloroplast(s), carbon dioxide and the photosynthetic action of illuminated — suspensions, BROWN AND FRANCK, 55

Clostridia, desthiobiotin and O-heterobiotin as growth factors for normal and degenerate —, PERLMAN, 79

Coenzyme I, estimation, KRISHNAN, 291

Compound  $C_{20}H_{20}O_8$ , isolation from the roots of *Solidago canadensis* L., HOUSTON AND BURRELL, 299

Conjugase, inhibition by proteins, HODSON, 309; — inhibitor (pteroylglutamic acid and sulfasuxidine induced leucopenia), SWENDSEID, BROWN, BIRD, AND HEINRICH, 367

Cow milk, see *Milk*

Cresolase, — from mushroom, MALLETTE, LEWIS, AMES, NELSON, AND DAWSON, 283

Crystallization, of beef peptomyosin, BOURDILLON, 61

Cyanide, — inhibition of retinal respiration, ROBBIE AND LEINFELDER, 437

Cytochrome oxidase, see *Malic dehydrogenase*

Cytosine, microbiological determination, MERRIFIELD AND DUNN, 339

## D

Deficiency, see *Pantothenic acid*, *Vitamin A*, *Zooperin*

Desthiobiotin, — as growth factor for clostridia, PERLMAN, 79

Diet (see also *Mouse*, *Pantothenic acid*, *Scorbut*, *Zooperin*); growth, reproduction, and lactation of rats fed purified rations, BOWLAND, ENSMINGER, CUNHA, 257; glycine and insulin in rats, CUNNINGHAM, BARNES, AND TODD, 403

$\alpha, \alpha$ -Di(glycylamino)propionic acid, enzymatic hydrolysis, GONÇALVES AND GREENSTEIN, 1

2,4-Dinitrotoluene, effect of fat and calories on the resistance of mice to —, CLAYTON AND BAUMANN, 415

Diseases, see *Achromotrichia*, *Carcinogenesis*, *Carcinoma*, *Leucopenia*, *Prothrombin*, *Virus*

Dog, see *Electrophoresis*

*Drosophila melanogaster*, adenosine triphosphate from — —, ALBAUM AND KLETZKIN, 333

Dye(s), metabolism of phthalein — by rat liver slices, ZIMMERBERG, 195

## E

- Eggs, enzymes of fresh hen —, LINEWEAVER, MORRIS, KLINE, AND BEAN, 443
- Electrophoresis, — of fibrinogen, AVERY AND MUNRO, 33; effect of repletion on the plasma proteins in the dog measured by —, CHOW, SEELEY, ALLISON, AND COLE, 69; — of lactoglobulins, BAIN AND DEUTSCH, 221
- Embriophyta sifonogama, alkaloid of (a plant belonging to) — —, SODI PALLARES AND GARZA, 275
- Endocrine, effect of extirpation of various — glands on the production of fatty liver, SHIPLEY, CHUDZIK, AND GYÖRGY, 301
- Enzymes(s) (see also *Adenylpyrophosphatase*, *Amylase*, *Aspartase*, *Coenzyme I*, *Conjugase*, *Glucose-6-phosphatase*, *Hyaluronidase*, *Malic dehydrogenase*, *Oxidase*, *Phosphatase*, *Tyrosinase*); enzymatic hydrolysis of  $\alpha,\alpha$ -Di(glycyl-amino) propionic acid, GONÇALVES AND GREENSTEIN, 1; mechanism of — action (XXXII), fat and sterol in *Fusarium lini* Bolley, *F. lycopersici*, and *F. solani* D<sub>2</sub> purple, NORD AND FIORE, 161; (catalase and saccharase) activity and nitrogen content of bacterial cells, VIRTANEN AND DELEY, 169; — and unsaturated ketones, GEIGER, 423; — of fresh hen eggs, LINEWEAVER, MORRIS, KLINE, AND BEAN, 443
- Epidermis, epidermal carcinogenesis in mice, ROBERTS AND CARRUTHERS, 239; calcium in normal —, hyperplastic —, and squamous cell carcinoma, LANSING, ROSENTHAL, AND AU, 361
- Ergosterol, see *Sterol*
- (XXXII), — and sterol in *fusariis*, NORD AND FIORE, 161
- Fatty, — liver, see *Liver*
- Fatty acid(s) (see also *Fat*); — transformations by anaerobic bacteria, ROSENFELD, 263
- Fibrinogen, chemical and electrophoretic studies of —, AVERY AND MUNRO, 33
- Flower of the heart, structure of the alkaloid of — —, SODI PALLARES AND GARZA, 275
- Fuchsin, spot tests with periodate —, HOTCHKISS, 131
- Fungi (see also *Fusarium*); antifungal activity of unsaturated ketones, GEIGER, 423
- Fusarium*, — *lini* Bolley, — *lycopersici*, — *solani* D<sub>2</sub> purple, fat and sterol in — — (mechanism of enzyme action, XXXII), NORD AND FIORE, 161

## G

- Galacturonic acid, color reaction, DISCHE, 409
- Glands, see *Endocrine*
- Glucose-6-phosphatase, — of liver, BROH-KAHN, MIRSKY, PERISUTTI, AND BRAND, 87
- Glutamic acid, influences on yeast growth (pantothenic acid studies, II), KING AND CHELDELIN, 231
- Goat lactoglobulin, BAIN AND DEUTSCH, 221
- Goldenrod, see *Solidago canadensis* L.
- Green turtle, see *Chelone mydas*
- Growth (see also *Bacteria*, *Streptomyces griseus*, *Yeast*); desthiobiotin and O-heterobiotin as — factors for clostridia, PERLMAN, 79; factors for —, reproduction, and lactation of rats fed purified rations, BOWLAND, ENSMINGER, AND CUNHA, 257

## H

- Hen, enzymes of fresh — eggs, LINEWEAVER, MORRIS, KLINE, AND BEAN, 443
- Fat (see also *2,4-Dinitrotoluene*, *Liver*, *Oil*); mechanism of enzyme action

- Heterobiotin, O- — as growth factor for clostridia, PERLMAN, 79
- Hexosemonophosphatase, the — system of liver, BROH-KAHN, MIRSKY, PERISUTTI, AND BRAND, 87
- Hog, — kidney conjugase, HODSON, 309
- Horse, — peptomyosin, BOURDILLON, 61
- Hyaluronidase, protein reserves and the production of — and antihyaluronidase, TOBIN, BERGENSTAHL, AND STEFFEE, 373
- Hydrolysis, enzymatic, see *Enzymes*
- I**
- Infectivity, see *Virus*
- Influenza, see *Virus*
- Inhibition (see also *Glutamic acid*, *Tomatine*); differentiation of bean trypsin inhibiting factors, BOWMAN, 109; inhibitory effect of proteins on the activity of hog kidney conjugase, HODSON, 309; cyanide — of retinal respiration, ROBBIE AND LEINFELDER, 437
- Insulin, — in rats, CUNNINGHAM, BARNES, AND TODD, 403
- Inulin, isolation of — and unidentified C<sub>20</sub> compound from the roots of *solidago canadensis* L., HOUSTON AND BURRELL, 299
- K**
- Ketones, antibacterial unsaturated —, GEIGER, 423
- Kidney, hog — conjugase, HODSON, 309
- L**
- Lactation, see *Growth*, *Malic dehydrogenase*
- Lactic acid, sulfur amino acid nutrition of — — producing bacteria, EVANS, 357
- Lactobacilli, microbiological determinations of cytosine, uracil and thymine by —, MERRIFIELD AND DUNN, 339; sulfur amino acid nutrition of —, EVANS, 357
- Lactoglobulins, studies on —, BAIN AND DEUTSCH, 221
- Lepidochelis olivacea Esch., mexican turtle oils (IV), — — GIRAL, 191
- Leucopenia, — by sulfasuccidine in the rat, pteroylglutamic acid, and conjugase, SWENDSEID, BROWN, BIRD, AND HEINRICH, 367
- Light, carbon dioxide and the photosynthetic activity of illuminated chloroplast suspensions, BROWN AND FRANCK, 55
- Liver, the hexosemonophosphatase system of (rat and rabbit) —, BROH-KAHN, MIRSKY, PERISUTTI, AND BRAND, 87; metabolism of phthalein dyes by rat — slices, ZIMMERBERG, 195; extirpation of endocrine glands and the production of fatty —, SHIPLEY AND CHUDZIK, AND GYÖRGY, 301
- Lutein tissue, malic dehydrogenase of — —, McSHAN, ERWAY, AND MEYER, 379
- M**
- Magnesium, activation of phosphatase by — ions, FISCHER AND GREP, 199
- Malic dehydrogenase, — — and cytochrome oxidase of lutein and other ovarian tissues during pregnancy and lactation, McSHAN, ERWAY, AND MEYER, 379
- Marine sediments, transformations of fatty acids buried in — —, ROSENFELD, 263
- Metabolism (see also *Amino acids*); — of various phthalein dyes by rat liver slices, ZIMMERBERG, 195
- Mexican, — turtle oils (I) GIRAL AND CASCAJARES, 177; (II), *Chelone mydas* Linn., GIRAL, GIRAL, AND GIRAL, 181; (III), *Caretta caretta* Linn., GIRAL AND MARQUEZ, 187; (IV), *Lepidochelis olivacea* Esch., GIRAL, 191
- Microchemistry, microchemical reaction resulting in staining of polysaccharide

- structures in fixed tissue preparations,  
HOTCHKISS, 131
- Milk, studies on lactoglobulins, BAIN  
AND DEUTSCH, 221
- Molds, see *Fusarium*
- Moisture, high- — storage and preser-  
vation of carotene in dehydrated alfalfa  
meal, MITCHELL, SCHRENK, AND KING,  
343
- Mouse, epidermal carcinogenesis in the  
—, ROBERTS AND CARRUTHERS, 239;  
ultrafilterable and non-ultrafilterable  
calcium in normal and carcinomatous  
epidermis in the —, LANSING, ROSEN-  
THAL, AND AU, 361; 2,4-dinitrotoluene  
in the —, CLAYTON AND BAUMANN,  
415
- Muscle, crystalline pepsin-resistant pro-  
tein from skeletal —, BOURDILLON, 61
- Mushroom, preparation of — tyrosinase,  
MALLETTE, LEWIS, AMES, NELSON,  
AND DAWSON, 233

## N

- Navy bean, see *Bean*
- Nitrogen, enzyme activity and — con-  
tent of bacterial cells, VIRTANEN AND  
DELEY, 169
- Nutrition, zoopherin, a nutritional fac-  
tor, ZUCKER, ZUCKER, BABCOCK, AND  
HOLLISTER, 115

## O

- Oil, see *Fat, Mexican* — —
- Olive, oxidase of —, CRUESS AND  
SUGIHARA, 39
- Ovarian tissue, malic dehydrogenase of  
— —, McSHAN, ERWAY, AND MEYER,  
379
- Oxidase, — of the olive, CRUESS AND  
SUGIHARA, 39
- Oxidation, oxygen consumption of Peni-  
cillium chrysogenum, WOLF, 143
- Oxygen, — absorption method for esti-  
mation of coenzyme I, KRISHNAN,  
291

## P

- Pantoic acid, influence on yeast growth  
(pantothenic acid studies, II), KING  
AND CHELDELIN, 231
- Pantothenic acid, — and sulfapyridine  
induced achromotrichia, WEST AND  
ELLIOTT, 47; — — studies (II), in-  
fluence of glutamic acid and pantoic  
acid on yeast growth, KING AND  
CHELDELIN, 231
- Penicillium chrysogenum, amino acid  
metabolism of — —, WOLF, 143
- Pepsin, crystalline — resistant protein  
from skeletal muscle, BOURDILLON, 61
- Peptomyosin, crystalline — BOURDILLON,  
61
- Periodic acid, spot tests with periodate-  
fuchsin, HOTCHKISS, 131
- Phenol red, see *Phthalein*
- Phenolphthalein, *Phthalein*
- Phosphatase, activation of purified alka-  
line —, FISCHER AND GREIF, 199
- Phosphorus, see *Adenosine triphosphate*,  
*Adenylpyrophosphatase*
- Photosynthesis, see *Light*
- Phthalcin, metabolism of — dyes by rat  
liver slices, ZIMMERBERG, 195
- Plasma (see also *Blood*); fibrinogen from  
human —, chemical and electro-  
phoretic studies, AVERY AND MUNRO,  
33; repletion of — proteins in the dog,  
CHOW, SEELEY, ALLISON, AND COLE,  
69
- Polysaccharides, see *Microchemistry*
- Potato, preparation of apyrase from —,  
KRISHNAN, 474
- Precipitation, — of fibrinogen, AVERY  
AND MUNRO, 33
- Pregnancy, see *Malic dehydrogenase*
- Protein(s) (see also *Lactoglobulin*, *Nitro-  
gen*); crystalline pepsin-resistant —  
from skeletal muscle, BOURDILLON, 61;  
repletion of plasma — in the dog,  
CHOW, SEELEY, ALLISON, AND COLE,  
69; zoopherin and animal — sources,  
ZUCKER, ZUCKER, BABCOCK, HOLLI-

- STER, 115; phosphatase and —, FISCHER AND GREEP, 199; effect of — on the activity of hog kidney conjugase, HODSON, 309; — reserves and the production of hyaluronidase and antihyaluronidase, TOBIN, BERGENSTAHL, AND STEFFEE, 373
- Prothrombin, adsorption by barium sulfate, ROSENFELD AND TUFT, 329
- Psalliota campestris*, see *Mushroom*
- Pteroylglutamic acid, — — and its hexaglutamyl conjugate; and leucopenia in the rat; effect of conjugase inhibitor, SWENDSEID, BROWN, BIRD, AND HEINRICH, 367
- Purple, see *Fusarium*
- Q**
- Quercetin, — and tomatine, MA AND FONTAINE, 399
- R**
- Rabbit, see *Liver*
- Radiation, effects of x- — on sodium thymus nucleate, TAYLOR, GREENSTEIN, AND HOLLAENDER, 19
- Rat(s) (see also *Liver*, *Pteroylglutamic acid*); pantothenate and sulfapyridine induced achromotrichia of the —, WEST AND ELLIOTT, 47; zoopherin, a nutritional factor for —, ZUCKER, ZUCKER, BABCOCK, AND HOLLISTER, 115; metabolism of phthalein dyes by — liver slices, ZIMMERBERG, 195; growth, reproduction, and lactation of —, BOWLAND, ENSMINGER, AND CUNHA, 257; fat in the — liver and the endocrine glands, SHIPLEY, CHUDZIK, AND GYÖRGY, 301; scorbutic symptoms in vitamin-A-deficient —, MAYER AND KREHL, 313; hyaluronidase in —, TOBIN, BERGENSTAHL, AND STEFFEE, 373; malic dehydrogenase and cytochrome oxidase of lutein in —, MCSHAN, ERWAY, AND MEYER, 379; insulin and glycine in —, CUNNINGHAM, BARNES, AND TODD, 403; cyanide inhibition of retinal respiration, ROBBIE AND LEINFELDER, 437
- Repletion, effect of — on the plasma proteins in the dog, CHOW, SEELEY, ALLISON, AND COLE, 69
- Reproduction, see *Growth*
- Retina, retinal respiration and cyanide, ROBBIE AND LEINFELDER, 437
- Rutin, — and tomatine, MA AND FONTAINE, 399
- S**
- S. cerevisiae*, see *Yeast*
- Saccharase, see *Enzymes*
- Scorbut, scorbutic symptoms in vitamin-A-deficient rats, MAYER AND KREHL, 313
- Skin, see *Epidermis*
- Solidago canadensis* L., inulin and an C<sub>20</sub> compound from the roots of —, HOUSTON AND BURRELL, 299
- Soybean, see *Bean*
- Spot tests, — — with periodate-fuchsin, HOTCHKISS, 131
- Staining, see *Microchemistry*
- Starch, hydrolysis by amylase, ROSE, 349
- Sterol, mechanism of enzyme action (XXXII), fat and — in fusariis, NORD AND FIORE, 161
- Stickland reaction, see *Bacteria*
- Storage, high-moisture — and preservation of carotene in dehydrated alfalfa meal, MITCHELL, SCHRENK, AND KING, 343
- Streptococci, microbiological determination of cytosine, uracil and thymine by —, MERRIFIELD AND DUNN, 399; sulfur amino acid nutrition of —, EVANS, 357
- Streptomyces griseus*, synthetic medium for — — and the production of streptomycin, THORNBERRY AND ANDERSON, 389
- Streptomycin, production of —, THORNBERRY AND ANDERSON, 389

Subtilin, isolation, LINEWEAVER, KLOSE,  
AND ALDERTON, 311

Sugar see *Carbohydrates*

Sulfapyridine, — induced achromotrichia  
and pantothenate, WEST AND ELLIOTT, 47

Sulfasuxidine, — induced leucopenia in  
the rat, and pteroylglutamic acid,  
SWENSEID, BROWN, BIRD, AND HEIN-  
RICH, 367

Sulfur, — amino acid nutrition of lactic  
acid producing bacteria, EVANS, 357

## T

Thymine, microbiological determination,  
MERRIFIELD AND DUNN, 339

Thymol blue, see *Phthalein*

Thymus nucleic acid, effects of x-radia-  
tion on the sodium salt of —, TAYLOR,  
GREENSTEIN, AND HOLLAENDER, 19

Tissues, see *Epidermis*, *Malic dehydro-  
genase*, *Microchemistry*

Tomatine, in vitro antibiotic activity  
of crystalline — toward candida  
albicans; antagonistic effect of rutin  
and quercetin, MA AND FONTAINE, 399

Trypsin, differentiation of bean — in-  
hibiting factors, BOWMAN, 109; —  
and phosphatase activity. FISCHER  
AND GREPP, 199

Tumors, see *Carcinogenesis*

Turtle, see *Mexican* . . .

Tyrosinase, preparation of mushroom —,  
MALLETTE, LEWIS, AMES, NELSON,  
AND DAWSON, 283

## U

Ultrafiltration, ultrafilterable and non-  
ultrafilterable calcium in normal hy-  
perplastic epidermis and squamous cell  
carcinoma, LANSING, ROSENTHAL, AND  
AU, 361

Uracil, microbiological determination,  
MERRIFIELD AND DUNN, 339

## V

Virus, thermal destruction of influenza A  
— infectivity, LAUFFER, CARNELLY,  
AND MACDONALD, 321

Vitamin, see *Pteroylglutamic acid*

Vitamin-A, scorbutic symptoms in — —  
deficient rats, MAYER AND KREHL, 313

## X

X-rays, see *Radiation*

## Y

Yeast (see also *Coenzyme I*); influence  
of glutamic acid and pantoic acid on  
— growth (pantothenic acid studies,  
II), KING AND CHELDELIN, 231  
Yoloxochitl, structure of the alkaloid of  
—, SODI PALLARES AND GARZA, 275

## Z

Zoopherin, — a nutritional factor,  
ZUCKER, ZUCKER, BABCOCK, AND  
HOLLISTER, 115



## INDEX OF BOOK REVIEWS

- |   |   |
|---|---|
| <p>ANDERSON, C. G., An Introduction to Bacteriological Chemistry (PORTER, J. R.), 479</p> <p>EUROPEAN ASSOCIATION OF CLINICAL PATHOLOGISTS, Recent Advances in Clinical Pathology (THANNHAUSER, S. J.), 315</p> <p>HUYBRECHTS, M., Le pH et sa mesure. Les potentials d'oxydo-reduction, le pH (MICHAELIS, L.), 315</p> <p>KAMEN, M. D., Radioactive Tracers in Biology (SOLOMON, A. K.), 153</p> <p>KOLTHOFF, I. M., AND STENGER, V. A., Volumetric Analysis, Vol. II, Titration Methods (YANOWSKY, L. K.), 319</p> <p>LACASSAGNE, A., Les cancers produits par des substances chimiques exogenes (WILLIAMS, R. J.), 320</p> <p>LACASSAGNE, A., Les cancers produits</p> | <p>par des rayonnements electromagnetiques (WACHTEL, H. K.), 480</p> <p>LAUFFER, M. A., Viruses (SMITH, K. M.), 316</p> <p>LIÉBECQ, C., Actualités Biochimiques, Conception Actuelle du Catabolisme de l'Hemoglobine (GRANICK, S.), 478</p> <p>LUCK, J. M., LORING, H. S., AND MACKINNEY, G., (ed.), Annual Review of Biochemistry (BELL, D. J.), 477</p> <p>MARKLEY, K. S., Fatty Acids, Their Chemistry and Physical Properties (WEITKAMP, A. W.), 318</p> <p>SORBA, M., Gonadotrophines et tumeurs testiculaires (WACHTEL, H. K.), 154</p> <p>THE ASSOCIATION OF VITAMIN CHEMISTS, Methods of Vitamin Assay (WILLIAMS, R. J.), 159</p> <p>VENDRYÈS, P., L'acquisition de la science (HOCKING, R.), 155</p> |
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